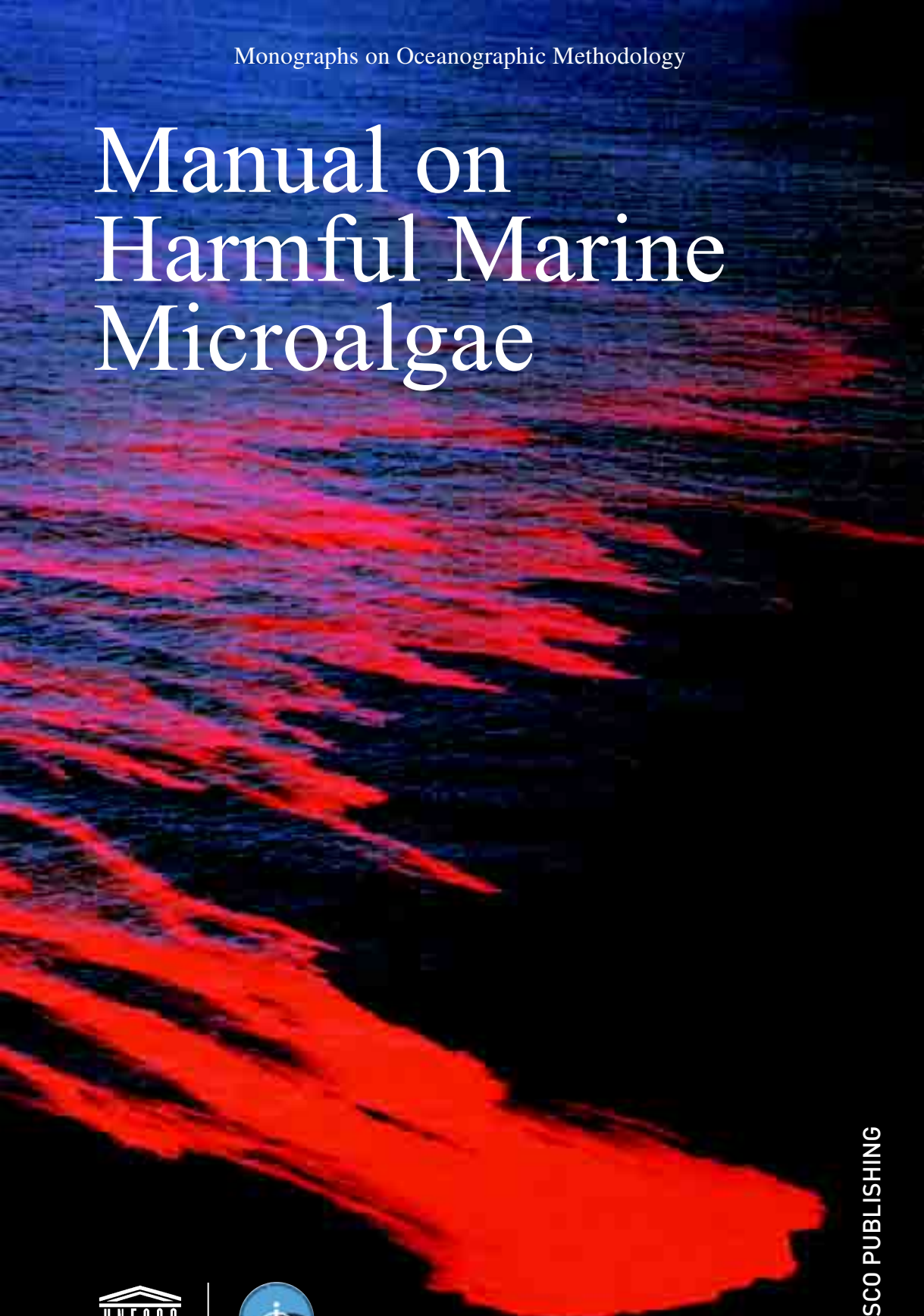
Monographs on Oceanographic Methodology

Manual on Harmful Marine Microalgae



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Manual on Harmful Marine Microalgae

Edited by G.M. Hallegraeff, D.M. Anderson and A.D. Cembella

Technical Director: H.O. Enevoldsen

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Preface

The Intergovernmental Oceanographic Commission (IOC) of UNESCO has for over a decade given attention to activities aimed at developing capacity in research and management of harmful microalgae. Due to the interdisciplinary nature of the topic, which covers taxonomy, toxin chemistry, monitoring and management, human health, etc., there was no comprehensive source of guidance with respect to both research and management methodologies until the first edition of this *Manual* was published in the 'IOC Manuals and Guides' series in 1995. The *Manual* was, and still is, intended to help fill this gap and to provide information and guidance in an easily accessible and low-cost format.

The first edition of the *Manual* was printed in 2,000 copies and has been one of the most sought-after IOC publications ever. As a consequence, the IOC Intergovernmental Panel on Harmful Algal Blooms (IPHAB) recommended that a second and expanded version be prepared, taking into account the experience and comments from the many users, including scientists, managers, course trainees and students.

One important task of the IOC and UNESCO is to synthesize the available field and laboratory research techniques for applications to help solve problems of society as well as to facilitate further research, especially systematic observations and data-gathering. The results include the publications in the 'IOC Manuals and Guides' series and the UNESCO series 'Monographs on Oceanographic Methodology'. The easy access to manuals and guides of this type is essential to facilitate knowledge exchange and transfer, the related capacity building, and for the establishment of ocean and coastal observations as envisaged in the Global Ocean Observing System.

The term 'harmful algae' is not a scientific but a societal one, determined by the increasingly detrimental effect of these organisms on national economies. One aim of supporting research and capacity building on harmful algae is to help mitigate their negative effect on fisheries, aquaculture, human health, recreation areas, ecosystems, etc. Understanding the causes of harmful algal events and developing contingency plans has direct links with other major areas of scientific and societal concern, including eutrophication, integrated coastal area management, fisheries management, and transfer of non-indigenous marine species. These links are important in addressing the problem of harmful algae adequately and in a way that will allow society to understand and appreciate the results of scientific advances within the field.

The United Nations Conference on Environment and Development (UNCED, 1992, in Rio de Janeiro) generated common goals and potential programmes related to sustainable development in the twenty-first century, *Agenda 21* and the two conventions on Climate Change and Biological Diversity fully recognized the need

for scientifically based information and methods for management, and specifically addressed the major areas of concern mentioned above. This *Manual*, in interaction with other IOC activities, should also be seen as a direct follow-up to UNCED and the implementation of *Agenda 21*.

The IOC is highly appreciative of the efforts of the more than 45 scientists who prepared the manuscripts for the first and second editions of the *Manual*, and wishes to express its particular thanks to Dr Gustaaf M. Hallegraeff, editor-in-chief, and Dr Donald M. Anderson and Dr Allan D. Cembella, co-editors, for their devoted involvement in this project over so many years.

The scientific opinions expressed in this work are those of the authors and are not necessarily those of UNESCO and the IOC. Equipment and materials have been cited as examples of those most currently used by the authors. Their inclusion does not imply that they should be considered as preferable to others available at that time or developed since.

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Patricio Bernal
Executive Secretary IOC

Contents

List of contributors

Per Andersen

Bio/consult as J. Ewaldsvej 42-44, 8230 Aabyhoej, Denmark
E-mail: pa@bioconsult.dk

Robert A. Andersen

Bigelow Laboratory for Ocean Sciences, PO Box 475, McKown Point, West Boothbay Harbor, ME 04575, USA
E-mail: Randersen@bigelow.org

Don M. Anderson

Biology Department, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA
E-mail: danderson@whoi.edu

Lorrie C. Backer

National Center for Environmental Health, Centers for Disease Control and Prevention, 1600 Clifton Rd, NE, MS E-23, Atlanta, GA 30333, USA
E-mail: lbacker@cdc.gov

Dan G. Baden

Department of Marine Biology and Fisheries, Rosenstiel School of Marine and Atmospheric Sciences, University of Miami, 4600 Rickenbacker Causeway, Miami, FL 33149-1098, USA
E-mail: baden@uncwil.edu

Juan Blanco

Centro de Investigaciónes Mariñas. Pedras de Corón, E- 36620 Vilanova de Arousa, Pontevedra, Spain.
E-mail: jblanco@cimacoron.org

Wayne W. Carmichael

Department of Biological Sciences-Process Development Centre for Biomolecules, Wright State University, 3640 Colonel Glen Highway, Dayton, OH 45435, USA
E-mail: wayne.carmichael@wright.edu

Edward J. Carpenter

Marine Sciences Research Center, State University of New York, Stony Brook, NY 11794, USA
E-mail: ecarpenter@ccmail.sunysb.edu

Allan D. Cembella

Alfred Wegener Institute
Department of Pelagic Ecosystems, Marine Chemistry and Marine Natural Products
Am Handelshafen 12
D-27570 Bremerhaven
Germany
E-mail: acembella@awi-bremerhaven.de

Gertrud Cronberg

Department of Ecology/Limnology, Ecology Building S-223 62, Lund, Sweden
E-mail: Gertrud.Cronberg@limnol.lu.se

Gregory J. Doucette

Marine Biotoxins Program, NOAA/National Ocean Service, 219 Fort Johnson Rd, Charleston, SC 29412 USA
E-mail: greg.doucette@noaa.gov

Henrik O. Enevoldsen

IOC Science and Communication Centre on Harmful Algae University of Copenhagen, Oster Farimagsgade 2 D, DK-1353 Copenhagen K, Denmark
E-mail: henrike@bot.ku.dk

Marisa L. Fernández

Technological Marine Centre, CETMAR Foundation, c/Eduardo Cabello s/n 36208, Vigo, Spain
E-mail: mfernandez@cetmar.org

Lora E. Fleming

Department of Epidemiology and Public Health, School of Medicine, University of Miami, 4600 Rickenbacker Causeway, Miami, FL 33149-1098, USA
E-mail: Lfleming @med.miami.edu

Peter J. S. Franks

Marine Life Research Group, Scripps Institution of Oceanography, University of California-0218, La Jolla, CA 92093-0218, USA
E-mail: franks@ucds.edu

Greta A. Fryxell

School of Biological Sciences, University of Texas, Austin, TX 78713-7640, USA
E-mail: gfryxell@mail.utexas.edu

Yasuwo Fukuyo

Asian Natural Environment Science Centre, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan
E-mail: ufukuyo@mail.ecc.u-tokyo.ac.jp

Ian Garthwaite

Toxinology, Food Science, AgResearch Ruakura, Private Bag 3123, East Street, Hamilton, New Zealand
E-mail: garthwaitei@agresearch.cri.nz

Robert R. L. Guillard

Bigelow Laboratory for Ocean Sciences, PO Box 475, McKown Point, West Boothbay Harbor, ME 04575, USA
E-mail: OMNET@bigelow.lab

Gustaaf M. Hallegraeff

School of Plant Science, University of Tasmania, GPO Box 252-55, Hobart, Tasmania 7001, Australia
E-mail: Hallegraeff@utas.edu.au

Yoshiaki Hara

Department of Biology, Faculty of Science, Yamagata University, Koshirakawa-cho 1-4-12, Yamagata 990, Japan
E-mail: hara@sci.kj.yamagata-u.ac.jp

Grethe R. Hasle

Biologisk Institutt, Avd. Marine Botanikk, Universitet i Oslo, PB 1069, Blindern, 0316 Oslo 3, Norway
E-mail: g.r.hasle@bio.uio.no

Christian Hummert

Universitat Jena, Institut für Ernahrung und Umwelt, Lebensmittelkunde, Dornburger Strasse 25, 07743 Jena, Germany
E-mail: hummert@mampf.ieu.uni-jena.de

Bruce A. Keafer

Biology Department, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA
E-mail: bkeafer@whoi.edu

Jacob Larsen

IOC Science and Communication Centre on Harmful Algae
Biological Institute, University of Copenhagen, Oster Farimagsgade 2D, DK-1353 Copenhagen K, Denmark
E-mail: jacobl@bot.ku.dk

Richard J. Lewis

Institute for Molecular Bioscience, University of Queensland, St Lucia, Queensland 4072, Australia
E-mail: ddrlewis@dingo.cc.uq.oz.au

Ben Long

School of Biomedical & Life Sciences, University of Surrey, Guilford, Surrey GU2 7XH, UK
E-mail: B.Long@surrey.ac.uk

Bernd Luckas

Universitat Jena, Institut für Ernahrung und Umwelt, Lebensmittelkunde, Dornburger Strasse 25, 07743 Jena, Germany
E-mail: B5belu@rz.uni-jena.de

Kazumi Matsuoka

Laboratory of Coastal Environmental Sciences, Faculty of Fisheries, Nagasaki University, 1-14 Bunkyo-Machi, Nagasaki 852-8521, Japan
E-mail: kazu-mtk@net.nagasaki-u.ac.jp

Øjvind Moestrup

Biological Institute, University of Copenhagen, Oster Farimagsgade 2 D, DK-1353 Copenhagen K, Denmark
E-mail: moestrup@bot.ku.dk

Steve L. Morton

NOS/NOAA, 219 Ft Johnson Road, Charleson, SC 29412, USA
E-mail: Steve.Morton@noaa.gov.

Yasukatsu Oshima

Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, 1-1 Tsutsumidori Amamiyamachi, Aoba-ku, Sendai 981, Japan
E-mail: oshimay@mail.cc.tohoku.ac.jp

Louis Peperzak

National Institute for Coastral and Marine Management/NICMM/RIKZ
P.O. Box 8039
NL-4330 EA Middelburg
The Netherlands
E-mail: L.Peperzak@rikz.rws.minvenw.nl

Michael A. Quilliam

Institute for Marine Biosciences, 1411 Oxford Street, Halifax, NS B3H 3Z1, Canada
E-mail: Michael.Quilliam@nrc.ca

Jack Rensel

Rensel Associates, 4209 234th Street N.E., Arlington, WA 98223, USA
E-mail: jackrensel@att.net

Lesley Rhodes

Cawthron Institute, Private Bag 2, Nelson, New Zealand
E-mail: lesley@cawthron.org.nz

Don Richard

CFIA Room 245
343 University Ave
Moncton, New Brunswick
Canada
E1C 9B6
E-mail: richarddj@inspection.gc.ca

Alan D. Rowan

Florida Department of Health, Epidemiology Bureau, 1317 Winewood Blvd, Tallahassee, FL 32399-0700, USA
E-mail: Alan_Rowan@doh.state.fl.us

Parke Rublee

University of North Carolina at Greensboro, PO Box 26174, Greensboro, NC 27402-6174, USA
E-mail: rublee@uncg.edu

Chris Scholin

Monterey Bay Aquarium Research Institute, PO Box 628, Moss Landing, CA 95039, USA
E-mail: scholin@mbari.org

Sandra E. Shumway

Natural Science Division, Southampton College, LIU, Southhampton, NY 11968, USA
E-mail: SShumway@southampton.liu.edu

Ted J. Smayda

Graduate School of Oceanography, University of Rhode Island, Kingston, RI 02881, USA
E-mail: tsmayda@gso.uri.edu

'Max' F. J. R. Taylor

Departments of Oceanography and Botany, University of British Columbia, 2075 Westbrook Place, Vancouver, BC V6T 1Z4, Canada
E-mail: maxt@mail.unixg.ubc.ca

Helge Thomsen

Danish Institute for Fisheries Research, Department of Marine Ecology and Aquaculture, Kavalergaarden 6, DK-2920 Charlottenlund, Denmark
E-mail: hat@dfu.min.dk

Jahn Throndsen

Biologisk Institutt, Avd. Marine Botanikk, Universitet i Oslo, PB 1069, Blindern, 0316 Oslo 3, Norway
E-mail: jahn.throndsen@bio.uio.no

Kirsten Todd

Cawthron Institute, 98 Halifax Street East, Private Bag 2, Nelson, New Zealand
E-mail: kirsten@cawthron.org.nz

Engel G. Vrieling

Department of Marine Biology, Center for Ecological and Evolutionary Studies, University of Groningen, Biological Center, PO Box 14, 9750 AA Haren, the Netherlands
E-mail: e.g.vrieling@biol.rug.nl

J. N. C. Whyte

Fisheries & Oceans Canada, Pacific Biological Station, 3190 Hammond Bay Road, Nanaimo, BC V9R 5K6, Canada
E-mail: whytei@pac.dfo-mpo.gc.ca

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The illustration of *Umezakia* natans has been printed by kind permission of M. Watanabe.

The authors acknowledge Karen Foster for her editorial assistance in writing Chapter 26.

Introduction

G. M. Hallegraeff

Research on harmful algal blooms (HABS) first emerged as a discipline in its own right at the First International Conference on Toxic Dinoflagellate Blooms which was held in Boston, Massachusetts, from 4 to 6 November 1974 (LoCicero, 1974). Of major concern at that time was the massive 1972 New England red tide caused by the toxic dinoflagellate *Alexandrium* (*Gonyaulax*) *tamarense*. This successful meeting was followed by two further International Conferences on Toxic Dinoflagellate Blooms held in Miami, Florida, in 1978 (Taylor and Seliger, 1979) and St Andrews, Canada, in 1985 (Anderson *et al.*, 1985). The First International Symposium on Red Tides in 1987 in Takamatsu, Japan (Okaichi *et al.*, 1989), broadened its scope to include bloom events caused by other algal groups (notably raphidophytes), and this tradition was followed with the Fourth to the Tenth International Conferences on Toxic Marine Phytoplankton held respectively in Lund, Sweden, in 1989 (Granéli *et al.*, 1990), Newport, USA, in 1991 (Smayda and Shimizu, 1993), Nantes, France, in 1993 (Lassus *et al.*, 1995), Sendai, Japan, in 1995 (Yasumoto *et al.*, 1996), Vigo, Spain, in 1997 (Reguera *et al.*, 1998), Hobart, Australia, in 2000 (Hallegraeff *et al.*, 2001) and Florida, USA, in 2002. A future conference in this series has been scheduled for South Africa in 2005. At the same time, a number of smaller regional meetings were convened to deal with exceptional plankton blooms in European coastal waters (Parker and Tett, 1987), the impact of algal blooms on aquaculture (Dale *et al.*, 1987), unusual 'brown tides' in Long Island, USA (Cosper *et al.*, 1989), tropical red tides of *Pyrodinium bahamense* in the Indo-West Pacific (White *et al.*, 1984; Hallegraeff and MacLean, 1989), newly recognized toxic diatom blooms in Canadian waters (Bates and Worms, 1989; Gordon, 1990) and an outbreak of neurotoxic shellfish poisoning in New Zealand (Jasperse, 1993). A NATO-ASI (North Atlantic Treaty Organization–Advanced Study Institute) workshop in Bermuda reviewed the physiological ecology of harmful algal blooms (Anderson *et al.*, 1998).

In 1989 the Fourth International Conference on Harmful Marine Phytoplankton reached a consensus 'that some human activities may be involved in increasing the intensity and global distribution of blooms' and recommended that 'international research efforts be undertaken to evaluate the possibility of global expansion of algal blooms and man's involvement in this phenomenon'. Subsequently, a number of new international programmes were created to study and manage harmful algal blooms and their links to environmental changes in a manner consistent with the global nature of the phenomena involved. The creation of a Harmful Algal Bloom programme by the Intergovernmental Oceanographic Commission of UNESCO has been one such initiative.

With increasing international collaboration came the unavoidable need for a standardization of methods. More importantly, it became clear that many developing countries had very limited access to outside literature and limited finances to buy books or to travel to overseas conferences. The first session of the IOC-FAO Intergovernmental Panel on Harmful Algal Blooms (Paris, 23–25 June 1992) therefore

agreed to support the production and free distribution to developing countries of a *Manual on Harmful Marine Microalgae*, which compiles widely different information on the taxonomy, toxicology and epidemiology of harmful algal blooms. As one of the early proponents of such a text, I accepted the responsibility as editor-in-chief (both an honour and a punishment!), capably assisted by Dr Don Anderson and Dr Allan Cembella and with expert technical assistance from Henrik Enevoldsen and staff of UNESCO Publishing. Authors were specifically instructed to target an audience in developing countries and therefore avoid unnecessarily sophisticated technologies.

In the choice of the title of this *Manual on Harmful Marine Microalgae*, we decided to abandon the term 'bloom' as some species such as *Chrysochromulina* and *Dinophysis* can cause serious problems even at moderate biomass levels. The term 'microalgae' is used instead of 'phytoplankton' in order to include problems caused by benthic species such as *Prorocentrum* and *Gambierdiscus*. A deliberate decision was made to omit problem growths of macroalgae. While some mention is made of toxic freshwater cyanobacteria (blue-green algae), the focus of this *Manual* is on marine microalgae.

Following a general introduction on harmful algal blooms, the *Manual* proper is composed of four parts: Methods, Taxonomy, Monitoring and Management, and Appendices. The Methods section covers oceanographic field-sampling techniques, algal-culture methods, cell counting, the use of antibody and DNA probes, instrumental toxin-analysis techniques for paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), ciguatoxins and cyanobacterial toxins, as well as biochemical methods and mammalian bioassays for selected algal toxins. Cyst methodologies are also included. The Taxonomy section starts with a general introduction on 'what is a species', followed by detailed accounts of the taxonomy of dinoflagellates, haptophytes, diatoms, raphidophytes, cyanobacteria and cysts. The Monitoring and Management section covers environmental monitoring, management of shellfish resources and finfish aquaculture, as well as epidemiology and public health. Lastly, in the Appendices, the reader will find a listing of algal-culture collections and addresses of international and regional agencies involved with harmful algal bloom studies. For some related topics the reader is also referred to the handbooks by Sournia (1978) (available stock almost exhausted), Parsons *et al.* (1984), and the conference proceedings mentioned above.

I hope that this *Manual* will help to alleviate economic hardships caused by harmful algal blooms, especially in developing countries, but also that it will foster further international research collaboration on this fascinating range of microorganisms which were already producing toxins hundreds of millions of years before humans turned to the oceans for aquaculture food production.

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Part I

Methods

1

Harmful algal blooms: a global overview

G. M. Hallegraeff

The microscopic planktonic algae of the world's oceans are critical food for filter-feeding bivalve shellfish (oysters, mussels, scallops, clams) as well as the larvae of commercially important crustaceans and finfish. In most cases, the proliferation of plankton algae (so-called 'algal blooms'; up to millions of cells per litre) therefore is beneficial for aquaculture and wild fisheries operations. However, in some situations algal blooms can have a negative effect, causing severe economic losses to aquaculture, fisheries and tourism operations and having major environmental and human health impacts. Among the 5,000 species of extant marine phytoplankton (Sournia *et al*., 1991), some 300 species can at times occur in such high numbers that they obviously discolour the surface of the sea (so-called 'red tides'), while only 80 or so species have the capacity to produce potent toxins that can find their way through fish and shellfish to humans (Table 1.1).

It is believed that the first written reference (1000 B.C.) to a harmful algal bloom appears in the Bible: ' . . . all the waters that were in the river were turned to blood. And the fish that was in the river died; and the river stank, and the Egyptians could not drink of the water of the river' (Exodus 7: 20–1). In this case, a non-toxic bloom-forming alga became so densely concentrated that it generated anoxic conditions resulting in indiscriminate kills of both fish and invertebrates. Oxygen depletion can be due to high respiration by the algae (at night or in dim light during the day) but more commonly is caused by bacterial respiration during decay of the bloom. Essentially non-toxic bloom formers sometimes can evoke major ecosystem impacts, however, and unsightly dead fish, slime and foam deter tourism and recreational activities.

One of the first recorded fatal cases of human poisoning after eating shellfish contaminated with dinoflagellate toxins was in 1793 when Captain George Vancouver and his crew landed in British Columbia in an area now known as Poison Cove. He noted that for local Indian tribes it was taboo to eat shellfish when the seawater became bioluminescent due to dinoflagellate blooms (Dale and Yentsch, 1978). The causative alkaloid toxins, now called paralytic shellfish poisons (PSP) (see Chapter 7), are so potent that a pinhead-size quantity (about 500 µg), which can easily accumulate in just one 100 g serving of shellfish, could be fatal to humans. On a global scale, close to 2,000 cases of human poisoning (15% mortality) through fish or shellfish consumption are reported each year and, if not controlled, the economic damage through reduced local consumption and reduced export of seafood products can be considerable. Whales and porpoises can also become victims when they receive toxins through the food chain via contaminated zooplankton or fish (Geraci *et al*., 1989). Poisoning of manatees by dinoflagellate brevetoxins contained in salps attached to seagrass (in Florida: Anderson and White, 1989) and of pelicans and

sealions by diatom domoic acid contained in anchovies has also been reported (in California: Scholin *et al*., 2000; Work *et al*., 1993).

TABLE 1.1 Different types of harmful algal bloom

1. Species that produce basically harmless water discolorations; however, under exceptional conditions in sheltered bays, blooms can grow so dense that they cause indiscriminate kills of fish and invertebrates through oxygen depletion. Examples: dinoflagellates *Akashiwo sanguinea*, *Gonyaulax polygramma*, *Noctiluca scintillans*, *Scrippsiella trochoidea*; cyanobacterium *Trichodesmium erythraeum*
2. Species that produce potent toxins that can find their way through the food chain to humans, causing a variety of gastrointestinal and neurological illnesses, such as:
• paralytic shellfish poisoning (PSP) (Examples: dinoflagellates *Alexandrium catenella*, *A. cohorticula*, *A. fundyense*, *A. fraterculus*, *A. leei*, *A. minutum*, *A. tamarense*, *Gymnodinium catenatum*, *Pyrodinium bahamense* var. *compressum*)
• diarrhetic shellfish poisoning (DSP) (Examples: dinoflagellates *Dinophysis acuta*, *D. acuminata, D. caudata, D. fortii, D. norvegica, D. mitra, D. rotundata*, *D. sacculus*, *Prorocentrum lima*)
• amnesic shellfish poisoning (ASP) (Examples: diatoms *Pseudo-nitzschia australis*, *P. delicatissima*, *P. multiseries*, *P. pseudodelicatissima*, *P. pungens* (some strains), *P. seriata*)
• ciguatera fish poisoning (CFP) (Examples: dinoflagellate *Gambierdiscus toxicus*,? *Coolia* spp.,? *Ostreopsis* spp.,? *Prorocentrum* spp.)
• neurotoxic shellfish poisoning (NSP) (Examples: dinoflagellate *Karenia brevis* (Florida), *K. papilionacea*, *K. selliformis*, *K. bicuneiformis* (New Zealand))
• cyanobacterial toxin poisoning (Examples: cyanobacteria *Anabaena circinalis* (freshwater), *Microcystis aeruginosa* (freshwater), *Nodularia spumigena*)
• estuarine associated syndrome (through aerosols from dinoflagellates *Pfiesteria piscicida*, *P. shumwayae)*
3. Species that are non-toxic to humans but harmful to fish and invertebrates (especially in intensive aquaculture systems) by damaging or clogging their gills. Examples: diatoms *Chaetoceros concavicorne, C. convolutus*; dinoflagellates *Karenia mikimotoi*, *K. brevisulcata*, *Karlodinium micrum*; prymnesiophytes *Chrysochromulina polylepis*, *Prymnesium parvum*, *P. patelliferum*; raphidophytes *Heterosigma akashiwo*, *Chattonella antiqua*, *C. marina*, *C. verruculosa*

The third type of harmful algal bloom has become apparent only as a result of our increased interest in intensive aquaculture systems for finfish. Some algal species can seriously damage fish gills, either mechanically or through production of hemolytic substances, whereas other algae kill fish through the production of extracellular neurotoxins. Whereas wild fish stocks have the freedom to swim away from problem areas, caged fish appear to be extremely vulnerable to such noxious algal blooms. In

1972 in Japan, a bloom of the raphidophyte flagellate *Chattonella antiqua* thus killed US$500 million worth of caged yellowtail fish in the Seto Inland Sea (Okaichi, 1989).

Table 1.1 summarizes the above three types of harmful algal bloom problems, together with representative examples of causative algal species, ranging from dinoflagellates, diatoms, prymnesiophytes and raphidophytes to cyanobacteria. Clinical symptoms of various types of fish and shellfish poisoning are listed in Table 1.2 and the diversity of chemical structures of algal toxins is discussed in Chapters 7–11. Unfortunately, there is no clear-cut correlation between algal concentrations and their potential harmful effects. Dinoflagellate species such as *Dinophysis*, *Alexandrium* and *Pyrodinium* can contaminate shellfish with toxins, even at very low cell concentrations. The prymnesiophyte *Chrysochromulina polylepis* produces only moderate biomass levels but has a very high toxic potency. Finally, the prymnesiophyte *Phaeocystis* is basically non-toxic but its nuisance value is caused by very high biomass levels.

1.1 Global increase of algal blooms

While harmful algal blooms, in a strict sense, are completely natural phenomena that have occurred throughout recorded history, in the past two decades the public health and economic impacts of such events appear to have increased in frequency, intensity and geographical distribution. One example, the increased global distribution of paralytic shellfish poisoning, is illustrated in Fig. 1.1. Until 1970, toxic dinoflagellate blooms of *Alexandrium (Gonyaulax) tamarense* and *Alexandrium (Gonyaulax) catenella* were only known from temperate waters of Europe, North America and Japan (Dale and Yentsch, 1978). By 1990, this phenomenon was well documented throughout the Southern Hemisphere, in South Africa, Australia, New Zealand, India, Thailand, Brunei, Sabah, the Philippines and Papua New Guinea. Other species of the dinoflagellate genus *Alexandrium*, such as *A. cohorticula* and *A. minutum*, as well as the unrelated dinoflagellates *Gymnodinium catenatum* and *Pyrodinium bahamense* var. *compressum* have now also been implicated. Unfortunately, there are very few long-term records of algal blooms at any single locality. Probably the best dataset refers to the concentration of PSP toxins (μg saxitoxin equivalent/100 g shellfish meat) in Bay of Fundy clams, which has been monitored by mouse bioassay since 1944 (White, 1987). Shellfish containing more than 80 μg PSP/100 g shellfish meat are considered unfit for human consumption. Fig. 1.2 shows evidence of a cyclic pattern of toxicity at this site with increased frequency of toxic blooms in the late 1940s, early 1960s, late 1970s and early 1980s, and possibly beginning again in the mid-1990s (not shown). The importance of such long-term datasets is discussed in Chapter 21.

The issue of a global increase in harmful algal blooms has been a recurrent topic of discussion at all major conferences dealing with harmful algal blooms (Anderson, 1989; Hallegraeff, 1993; Smayda, 1990). Four explanations for this apparent increase in algal blooms have been proposed: increased scientific awareness of toxic species; increased utilization of coastal waters for aquaculture; stimulation of plankton blooms by cultural eutrophication and/or unusual climatological conditions; and transportation of dinoflagellate resting cysts either in ships' ballast water or associated with translocation of shellfish stocks from one area to another.

TABLE 1.2 Clinical symptoms of various types of fish and shellfish poisoning

Paralytic shellfish poisoning (PSP)	**Diarrhetic shellfish poisoning (DSP)**	**Amnesic shellfish poisoning (ASP)**	**Neurotoxic shellfish poisoning (NSP)**	**Ciguatera fish poisoning**
Causative organism				
Alexandrium catenella	*Dinophysis acuminata*	*Pseudo-nitzschia multiseries;* *Pseudo-nitzschia pungens* (some strains)	*Karenia brevis* (Florida); *K. papilionacea* *K. selliformis* *K. bicuneiformis* (New Zealand)	*Gambierdiscus toxicus*
Alexandrium minutum	*Dinophysis acuta*	*Pseudo-nitzschia pseudodelicatissima*		*?Ostreopsis siamensis*
Alexandrium tamarense	*Dinophysis fortii*	*Pseudo-nitzschia australis*		*?Coolia monotis*
Gymnodinium catenatum	*Dinophysis norvegica*	*Pseudo-nitzschia seriata*		
Pyrodinium bahamense var. *compressum*	*Prorocentrum lima*	*Pseudo-nitzschia delicatula*		
Symptoms				
Mild case				
Within 30 min: tingling sensation or numbness around lips, gradually spreading to face and neck; prickly sensation in fingertips and toes; headache, dizziness, nausea, vomiting, diarrhoea.	After 30 min to a few hours (seldom more than 12 hours): diarrhoea, nausea, vomiting, abdominal pain.	After 3–5 hours: nausea, vomiting, diarrhoea, abdominal cramps.	After 3–6 hours: chills, headache, diarrhoea; muscle weakness, muscle and joint pain; nausea and vomiting	Symptoms develop within 12–24 hours of eating fish. Gastrointestinal symptoms: diarrhoea, abdominal pain, nausea, vomiting.

Extreme case				
Muscular paralysis; pronounced respiratory difficulty; choking sensation; death through respiratory paralysis may occur within 2–24 hours after ingestion.	Chronic exposure may promote tumour formation in the digestive system.	Decreased reaction to deep pain; dizziness, hallucinations, confusion; short-term memory loss; seizures	Paraesthesia; altered perception of hot and cold; difficulty in breathing, double vision, trouble in talking and swallowing	Neurological symptoms: numbness and tingling of hands and feet; cold objects feel hot to touch; difficulty in balance; low heart rate and blood pressure; rashes. In extreme cases, death through respiratory failure.
Treatment				
Patient has stomach pumped and is given artificial respiration. No lasting effects.	Recovery after three days, irrespective of medical treatment.			No antitoxin or specific treatment is available. Neurological symptoms may last for months or years. Calcium and mannitol may help to relieve symptoms.

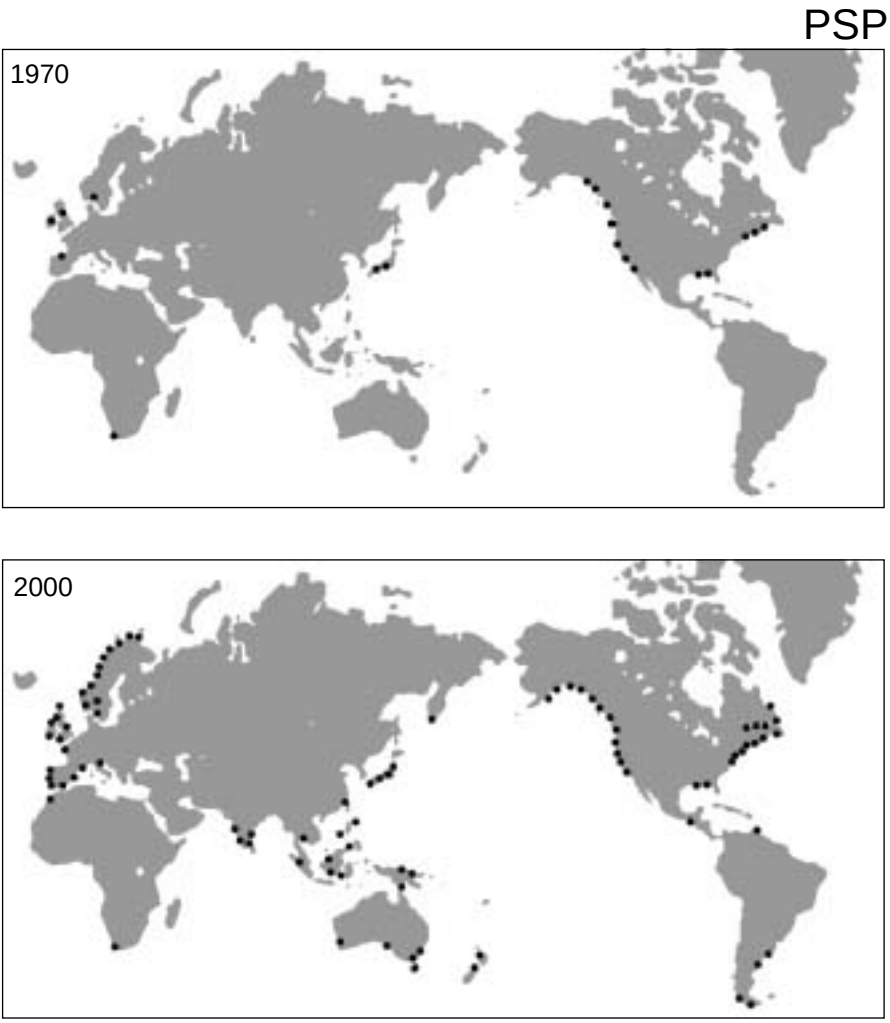

Figure 1.1
Known global distribution of paralytic shellfish poisoning (PSP) in 1970 and 2000.

1.2 Increased scientific awareness of toxic species

Reports of harmful algal blooms, associated human illnesses or damage to aquaculture operations are receiving increased attention in the press, electronic media and scientific literature. Fig. 1.3 illustrates the doubling of annual literature on algal blooms every two to two-and-a-half years. As a result, more and more researchers are now surveying their local waters for the causative organisms. Increased reports

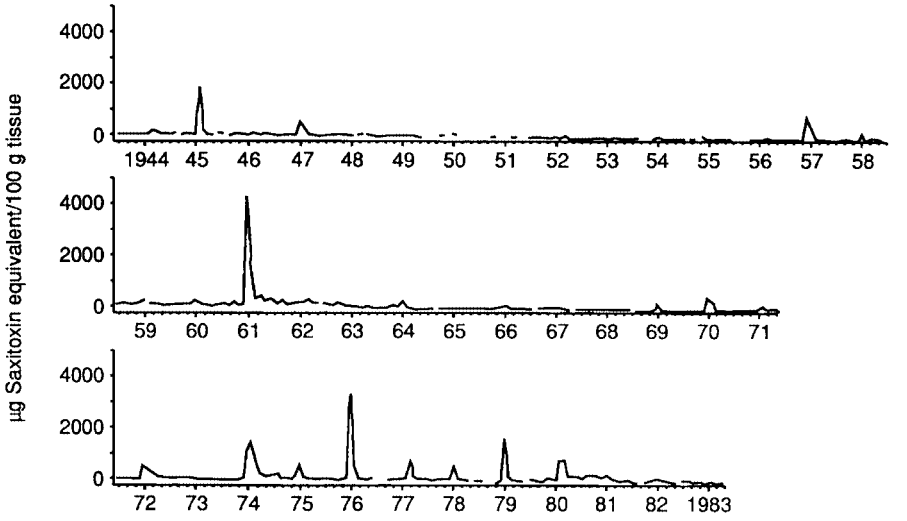

Figure 1.2
Concentration of PSP toxins in Bay of Fundy clams (µg saxitoxin equivalent/100 g tissue) in 1944–1983.
Source: White (1987).

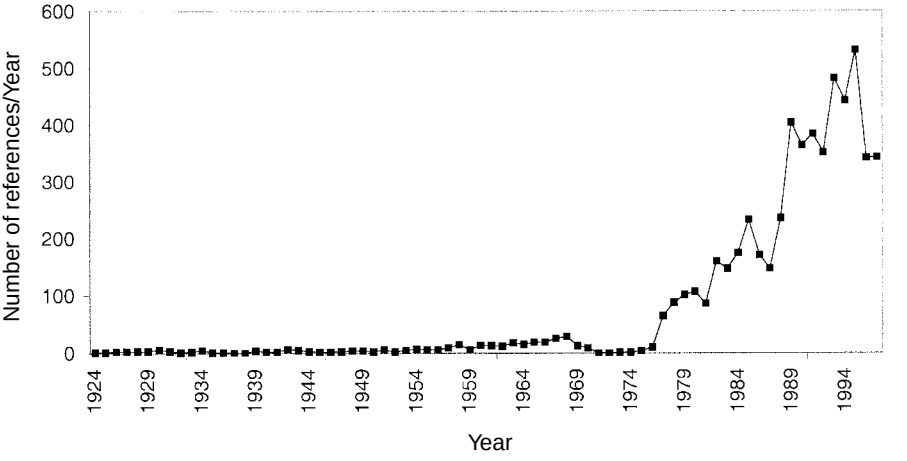

Figure 1.3
Growth of literature on harmful algal blooms, illustrated by analysing some 6,000 HAB publications from Aquatic Sciences and Fisheries Abstracts (ASFA) from about 1978 to 1998, with pre-1970 literature based on University of Copenhagen records.
Source: data courtesy of Gert Hansen.

on the occurrence of dinoflagellates of the genus *Alexandrium* are a good example. Until 1988, the type species *A. minutum* was only known from Egypt (Halim, 1960); it has now been reported from Australia, Ireland, France, Spain, Portugal, Italy, Turkey, the east coast of North America, Thailand, New Zealand, Taiwan and Japan (Hallegraeff *et al*., 1991; Yuki, 1994). Other examples are the recent description of the newly recognized phenomena of diarrhetic shellfish poisoning (since 1976), amnesic shellfish poisoning (since 1987) and azaspiracid poisoning (AZP, since 2000, so far only documented from Ireland, England and Norway).

1.2.1 Diarrhetic shellfish poisoning (DSP)

This phenomenon was first documented in 1976 from Japan where it caused major problems for the scallop fishery (Yasumoto *et al*., 1978). The first dinoflagellate to be implicated was *Dinophysis fortii* (in Japan), soon followed by *D. acuminata* (in Europe), *D. acuta, D. norvegica* (in Scandinavia), *D. mitra, D. rotundata* and the benthic dinoflagellate *Prorocentrum lima*. Between 1976 and 1982, some 1,300 DSP cases were reported in Japan, in 1981 more than 5,000 cases were reported in Spain and in 1983 some 3,300 cases were reported in France. In 1984 in Sweden, DSP problems caused a shutdown of the mussel industry for almost a year. The clinical symptoms of DSP (Table 1.2) may often have been mistaken for those of bacterial gastric infections and the problem may be much more widespread and serious than previously thought. Unlike PSP, no human fatalities have ever been reported and patients usually recover within three days. However, some of the polyether toxins involved (okadaic acid, dinophysis toxin-1; see Chapter 8) may promote stomach tumours (Suganuma *et al*., 1988) and thus produce chronic problems in shellfish consumers. Shellfish containing more than 2 µg okadaic acid and/or 1.8 µg dinophysis toxin-1 per gram of hepatopancreas are considered unfit for human consumption (Lee *et al*., 1987). Increasing problems caused by pectenotoxins have also been noted. The known global distribution of DSP (Fig. 1.4) includes Japan, Europe, Chile, Thailand, Canada (Nova Scotia), Australia and New Zealand.

1.2.2 Amnesic shellfish poisoning (ASP)

This phenomenon was first recognized in 1987 in Prince Edward Island, Canada, where it caused three deaths and 105 cases of acute human poisoning following the consumption of blue mussels. The symptoms (Table 1.2) include abdominal cramps, vomiting, disorientation and memory loss (amnesia). Most unexpectedly, the causative toxin (the excitatory amino acid domoic acid; see Chapter 9) is produced by a diatom and not by a dinoflagellate. Shellfish containing more than 20 µg domoic acid per gram of shellfish meat are considered unfit for human consumption. The diatom species *Pseudo-nitzschia australis* (= *N. pseudoseriata*), *P. delicatissima, P. multiseries, P. multistriata, P. pseudodelicatissima, P. seriata* and occasionally *P. fraudulenta, P. pungens* and *P. turgidula* have been implicated (Bates *et al*., 1989; Garrison *et al*., 1993; Martin *et al*., 1990; Rhodes *et al*., 1998). To date, reports of domoic acid in seafood products have been mainly confined to North America (Bay of Fundy, California, Oregon, Washington, Alaska) and Canada (Prince Edward Island, British Columbia), whereas only insignificant concentrations have been detected in other parts of the world such as Europe, Australia, Japan and New Zealand (Fig. 1.5). Of further concern is the demonstration of domoic acid production by *Nitzschia navis-varingica* from a tropical shrimp aquaculture pond (Lundholm and Moestrup, 2000).

1.3 Increased utilization of coastal waters for aquaculture

With increased problems of overfishing of coastal waters, more and more countries are looking towards aquaculture as an alternative. Indeed, fisheries scientists predict that, within the next 10–20 years, the increasing value of world aquaculture production may well approach the decreasing value of the total catch of wild fish and shellfish. Aquaculture operations act as sensitive 'bioassay systems' for harmful algal

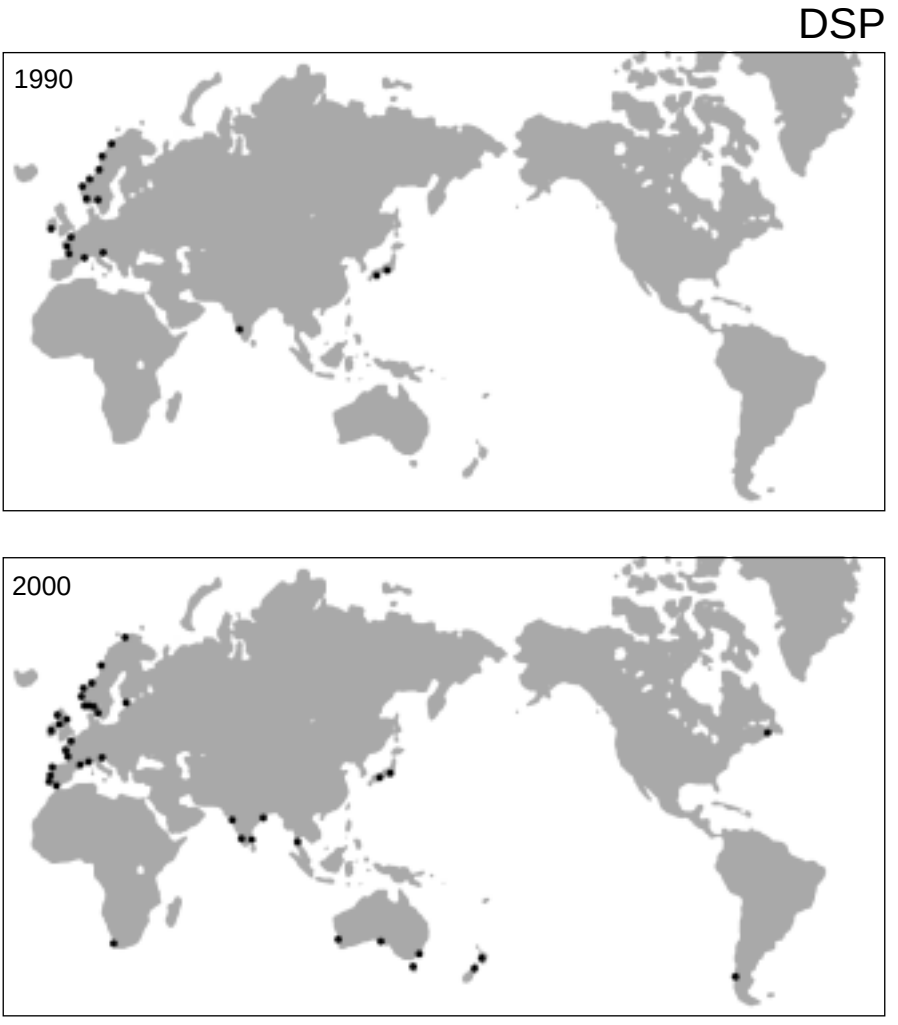

Figure 1.4
Known global distribution of diarrhetic shellfish poisoning (DSP) in 1990 and 2000.

species and can bring to light the presence in water bodies of problem organisms not previously known to exist there. The increase in shellfish farming worldwide is leading to more reports of paralytic, diarrhetic, neurotoxic or amnesic shellfish poisoning. On the other hand, increased finfish culture is drawing attention to algal species which can cause damage to the fishes' delicate gill tissues.

In fish pens in British Columbia, deaths of lingcod, sockeye, coho, chinook and pink salmon have been caused by dense concentrations (5,000 cells per litre) of the diatoms *Chaetoceros convolutus* and *C. concavicornis*. The diatom's long hollow

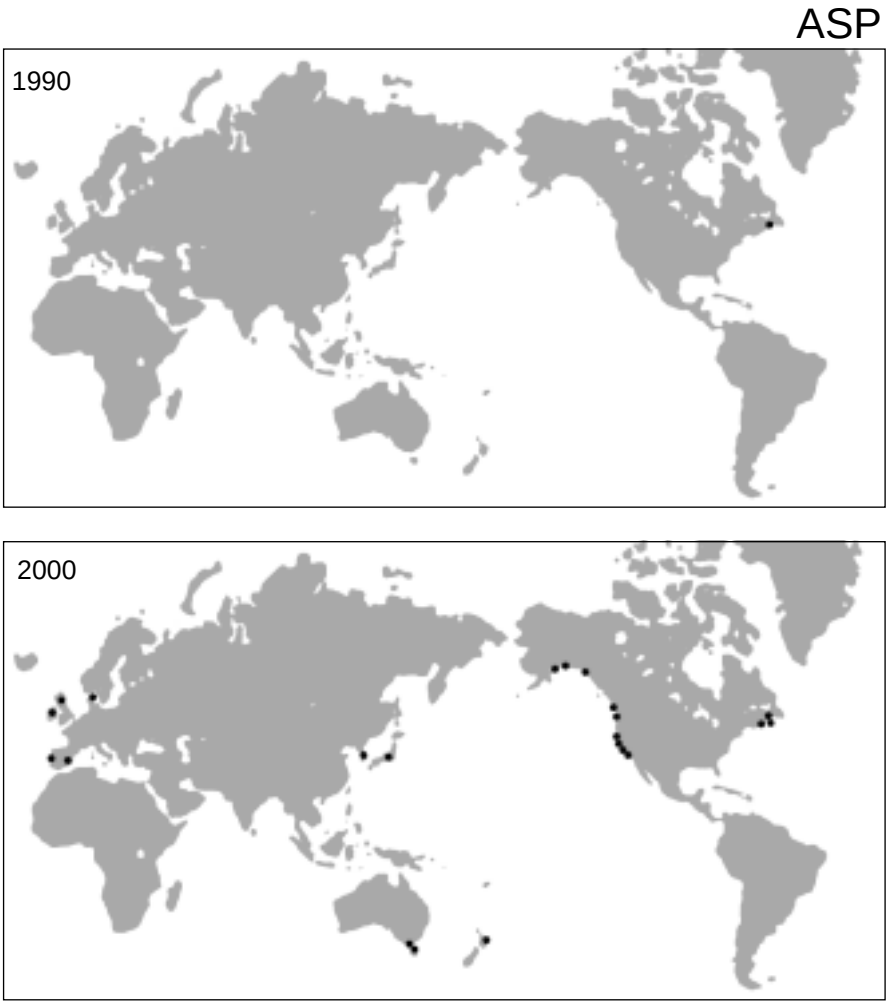

Figure 1.5
Known global distribution of amnesic shellfish poisoning (ASP) in 1990 and 2000.

spines (setae) are studded with smaller barbs along their length. The setae can break off and penetrate the gill membranes of fish, with the smaller barbs preventing them from coming out. Fish death may be caused by capillary haemorrhage, dysfunction of gas exchange at the gills, suffocation from an overproduction of mucus, or even from secondary infection of the damaged tissue (Bell, 1961; Rensel, 1993; Yang and Albright, 1992).

A more widespread problem for fish farmers is the production by various algal groups of fatty acids or galactolipids which damage the epithelial tissues of the gills. In experimental assay systems these substances destroy red blood cells and therefore have been provisionally termed 'hemolysins' (Yasumoto *et al*., 1990). Algal species as diverse as the raphidophytes *Heterosigma akashiwo* and *Chattonella antiqua/marina* (see Chapter 18), the prymnesiophytes *Chrysochromulina polylepis* and *Prymnesium parvum* (see Chapter 16), and the dinoflagellate *Karenia mikimotoi* (= *Gymnodinium nagasakiense;* see Chapter 15) have been implicated. *Heterosigma* has killed caged fish in Japan, Canada, Chile and New Zealand, whereas *Chattonella* is a fish killer known from Japan (Seto Inland Sea), South East Asia, Australia and Europe. With these two raphidophyte flagellates, physical clogging of gills by mucus excretion or gill damage by hemolytic substances or the production of oxygen radicals, free fatty acids and breve-like neurotoxins may be involved. In January 1989, a *Heterosigma* bloom in Big Glory Bay, Stuart Island (New Zealand), killed cage-reared chinook salmon worth NZ$12 million (Chang *et al*., 1990), and *Chattonella marina* killed Aus$45 million of cultured blue-fin tuna in South Australia in 1996 (Hallegraeff *et al*., 1998). The two prymnesiophyte flagellates *Chrysochromulina* and *Prymnesium* produce substances that affect gill permeability, which leads to a disturbed ion balance. Toxicity by these species is promoted by phosphorus deficiency. A massive bloom (60,000 km^2; 10^7 cells l^{-1}) of *Chrysochromulina polylepis* occurred in May–June 1988 in the Skagerrak, the Kattegat, the Belt and the Sound between Denmark, Norway and Sweden (Rosenberg *et al*., 1988). The deaths of 900 tons of fish, including cod, salmon and trout, occurred due to damage of gill membranes that produced a lethal increase in the chloride concentration in the blood; fish cages moved into less saline fjords were therefore less affected. *Prymnesium parvum* has caused mortality of *Tilapia* fish in brackish water culture ponds in Israel (Shilo, 1981), as well as mortality of salmon and rainbow trout in net-pens in Norway. However, probably the greatest problem for Norwegian fish farms are blooms of the unarmoured dinoflagellate *Karenia mikimotoi* (as *Gyrodinium aureolum*) (first reported in 1966; Tangen, 1977). Similar dinoflagellates are common in Ireland and Scotland, as well as Japan and Korea. Characteristic histopathological symptoms in fish are a severe necrosis and sloughing of epithelial tissues of the gills and digestive system (Roberts *et al*., 1983). A bloom of related gymnodinioid dinoflagellate species in Hong Kong waters in April 1998 caused over US$20 million damage to finfish aquaculture.

Sophisticated monitoring systems using buoys with fibre-optical sensors and data transfer by satellite (the MARINET system) are in place on the Norwegian coast to allow cages to be towed away from bloom-affected areas. During the 1988 *Chrysochromulina* bloom, more than 26,000 tons of fish in 1,800 cages were thus moved from their permanent site into inland fjords. Fish losses in cages can also be reduced by not feeding the fish, as feeding attracts them to the surface and increases oxygen demand. In some cases, pumping of water to dilute the algal concentration, the administration to fish of mucolytic agents or immediate harvesting of marketable

fish before they can be killed by algal blooms may also be an option. The hemolytic toxins do not accumulate in fish flesh. Virtually all algal blooms, even of non-toxic species, reduce the fishes' appetite and reduced oxygen concentrations stress the fish and make them more vulnerable to disease (see Chapter 25).

Finally, ichthyotoxic 'ambush predator' dinoflagellates *Pfiesteria piscicida* and *P. shumwayae* were first recognized in North Carolina in 1991 and later in Chesapeake Bay (Burkholder *et al.*, 1992). Their ephemeral presence (cysts germinate in the presence of live fish and encyst again after fish death) may explain many mysterious fish kills along the south-east coast of the USA and has also been associated with human health impacts (estuarine associated syndrome). *Pfiesteria* has now also been documented from northern Europe, New Zealand and Australia.

1.4 Increase of algal blooms by cultural eutrophication

While some organisms such as the dinoflagellates *Karenia (Gymnodinium) brevis, Alexandrium, Dinophysis* and *Pyrodinium* appear to be unaffected by coastal nutrient enrichments, many other algal bloom species appear to be stimulated by 'cultural eutrophication' from domestic, industrial and agricultural wastes. Fig. 1.6 illustrates an 8-fold increase in the number of red tides per year in Tolo harbour, Hong Kong, in the period 1976–1986 (Lam and Ho, 1989). This increase (mainly *Karenia mikimotoi, Gonyaulax polygramma, Noctiluca scintillans* and *Prorocentrum minimum = cordatum)* shows a striking relationship with the 6-fold increase in human population in Hong Kong and the concurrent 2.5-fold increase in nutrient loading, mainly contributed by untreated domestic and industrial waste. Red-tide events in Hong Kong harbour were less frequent in 1989–1997 until the major bloom year of 1998. A similar experience was noted in the Seto Inland Sea, one of the major fish-farm areas in Japan (Okaichi, 1989) (Fig. 1.7). Between 1965 and 1976, the number of

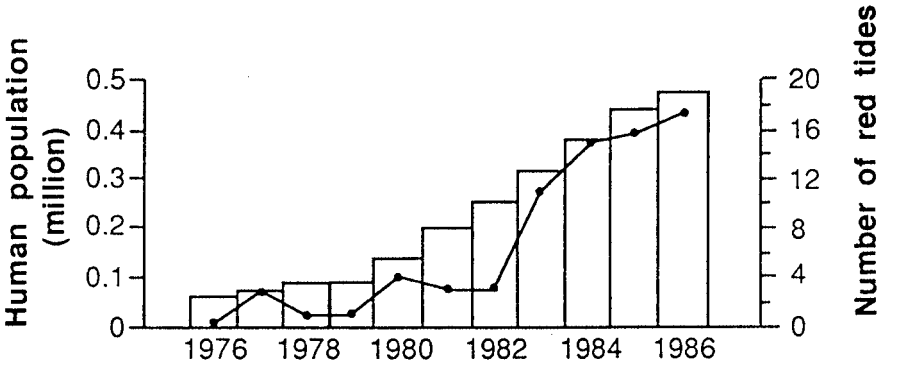

Figure 1.6
Correlation between the number of red-tide outbreaks per year in Tolo Harbour (continuous line) and the increase in the human population in Hong Kong (bar diagram), 1976–1986.
Source: Lam and Ho (1989).

confirmed red-tide outbreaks (mainly *Chattonella antiqua*, since 1964; and *Karenia mikimotoi*, since 1965) progressively increased 7-fold, concurrent with a 2-fold increase in the COD (chemical oxygen demand) loading, mainly from untreated sewage and industrial waste from pulp and paper factories. During the most severe outbreak in 1972, a *Chattonella* red tide killed 14 million cultured yellow-tail fish. Effluent controls were then initiated to reduce the chemical oxygen demand loading by about half, to introduce secondary sewage treatment and to remove phosphate from household detergents. Following a time-lag of four years, the frequency of red-tide events in the Seto Inland Sea then decreased about 2-fold to a more stationary level.

A similar pattern of a long-term increase in nutrient loading of coastal waters is evident for the North Sea in Europe (Smayda, 1990) (Fig. 1.8). Since 1955, the phosphate loading of the River Rhine has increased 7.5-fold, whereas nitrate levels have increased 3-fold. This has resulted in a significant 6-fold decline in the Silicon: phosphorus ratio, because long-term reactive silicate concentrations (a nutrient derived from natural land weathering) have remained constant. More recently, improved wastewater treatment has been causing increases in the ammonia:nitrate ratio of River Rhine discharge (Riegman *et al*., 1992). The nutrient composition of treated wastewater is never the same as that of the coastal waters into which it is being discharged. Furthermore, atmospheric deposition of nitrogen also needs to be included in budgets of anthropogenic nutrient input. There is considerable concern (Officer and Ryther, 1980; Ryther and Dunstan, 1971; Smayda, 1990) that such altered nutrient ratios in coastal waters may favour blooms of nuisance flagellate species which replace the normal spring and autumn blooms of siliceous diatoms.

The remarkable increase in foam-producing blooms of the prymnesiophyte *Phaeocystis pouchetii*, which first appeared in Dutch coastal waters in 1978, is probably the best-studied example of this phenomenon (Lancelot *et al*., 1987). The 1988 bloom in the Kattegat of the prymnesiophyte *Chrysochromulina polylepis*,

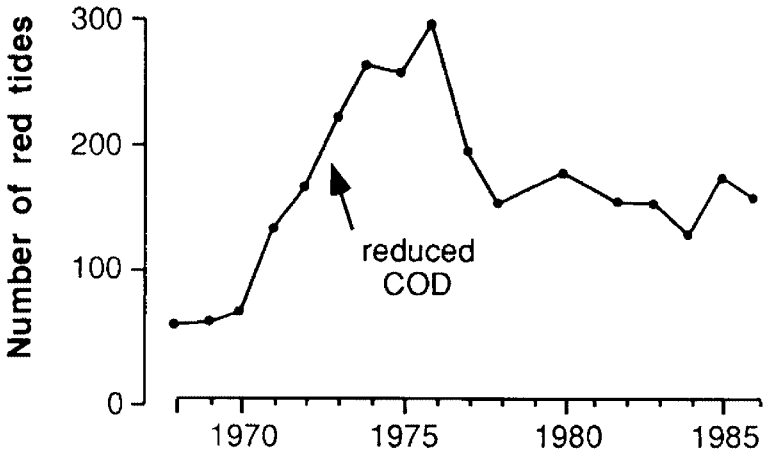

Figure 1.7
Long-term trend in the frequency of red-tide outbreaks in the Seto Inland Sea, Japan, 1965–1986. COD: chemical oxygen demand.
Source: Okaichi (1989).

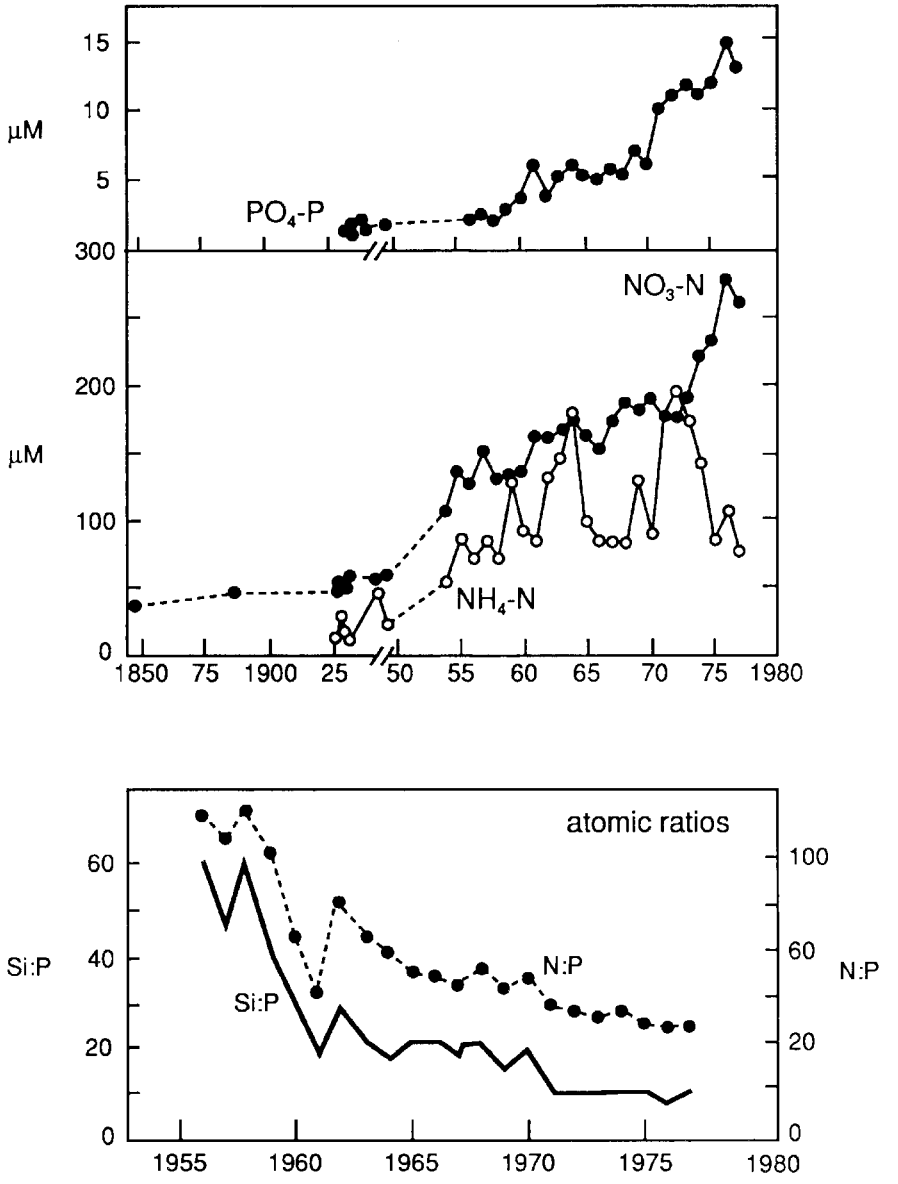

Figure 1.8
Long-term trend in the phosphate, nitrate and ammonia loading of the River Rhine (above) and concurrent changes in the N:P and Si:P nutrient ratios (below).
Source: Smayda (1990), using data by Van Bennekom and Salomons (1981).

not unusual in terms of biomass but rather in terms of its species composition and toxicity, has been related to a change in the nutrient-status from nitrogen- to phosphorus-limitation (Maestrini and Granéli, 1991). As in Hong Kong and Japan, several North European countries have now agreed to reduce phosphate and nitrate discharges by 50% in the next several years, but their efforts will almost certainly be in vain if neighbours continue polluting. Furthermore, such indiscriminate reductions in nutrient discharges are not addressing the problem of changing nutrient ratios of coastal waters. Changed patterns of land use, such as deforestation, can also cause shifts in phytoplankton species composition by increasing the concentrations of humic substances in land runoff. Acid precipitation can further increase the mobility of humic substances and trace metals in soils. Experimental evidence from Sweden indicates that river water draining from agricultural soils (rich in N and P) stimulates diatom blooms but that river water draining from forest areas (rich in humic and fulvic acids) can stimulate dinoflagellate blooms of species such as *Prorocentrum minimum* (Granéli and Moreira, 1990). Agricultural runoff of phosphorus can also stimulate cyanobacterial blooms, for example of *Nodularia spumigena* in the Baltic Sea and in the Peel-Harvey Estuary, Australia (Fig. 1.9). These species produce hepatotoxic peptides (*Nodularia, Microcystis*) and neurotoxic alkaloids (*Anabaena, Aphanizomenon)* which can kill domestic and wild animals drinking from the shores of eutrophic ponds, lakes and reservoirs (for example, during a 1,000 km long *Anabaena circinalis* bloom in the Darling River, Australia, in 1991). Toxicity problems from freshwater cyanobacteria have been documented from Australia, Bangladesh, China, Europe (12 countries), India, Israel, Japan, Latin America, North America, South Africa, Thailand and the former Soviet Union (Carmichael, 1989). The toxins can accumulate in the digestive system of shellfish (Falconer *et al*., 1992) but contamination of drinking water with teratogens and tumour promoters is a more common public health risk. Human fatalities have resulted when microcystin-contaminated lake water was offered to patients in a haemodialysis clinic (Pouria *et al*., 1998). A neurotoxic factor has also been associated with some strains of the

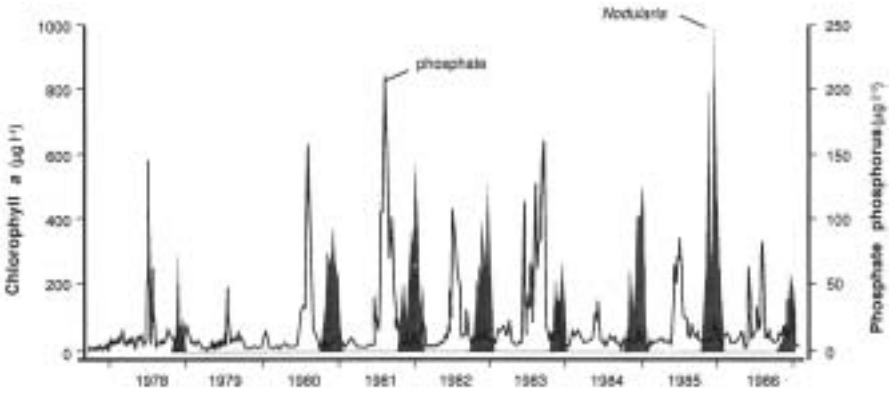

Figure 1.9
Relationship between *Nodularia spumigena* cyanobacterial blooms (as chlorophyll concentration) in the Peel-Harvey Estuary, Australia, and its relationship to riverine phosphate loading from agricultural runoff.
Source: Hillman *et al.* (1990).

common marine, bloom-forming tropical cyanobacterium *Trichodesmium thiebautii* (Hawser *et al*., 1991: see Chapter 11).

A much more complex 'cultural eutrophication' scenario has emerged in coastal waters of New Jersey, New York and Rhode Island, USA, where an unusual 'brown tide' (caused by the chrysophyte picoplankton *Aureococcus anophagefferens*) has been related to the discharge of chelators (such as citric acid) in detergents and lawn treatments, together with a suppression of zooplankton grazing by pesticides (Cosper *et al*., 1989, 1993). This bloom was responsible for a reduction in the extent and biomass of eelgrass beds and caused starvation and recruitment failure in commercial scallop populations. Suppression of zooplankton grazing by the overexploitation of piscivorous fish can similarly release HAB species from grazing. Eutrophication problems like this cannot be readily diagnosed by routine monitoring programmes that focus on macronutrients or algal chlorophyll biomass alone (see Chapter 21).

1.4.1 Ciguatera fish poisoning and coral reef disturbance

Ciguatera is a tropical fish food-poisoning syndrome well known from coral reef areas in the Caribbean, Australia, and especially French Polynesia (Fig. 1.10). Humans consuming contaminated fish such as red bass, chinaman fish, moray eel, and paddle tail can suffer from gastrointestinal and neurological illnesses and in extreme cases can die from respiratory failure (Table 1.2; Gillespie *et al*., 1986). The causative organisms are benthic dinoflagellates such as *Gambierdiscus toxicus*, and possibly *Ostreopsis siamensis*, *Coolia monotis* and related species, that live in epiphytic association with bushy red, brown and green seaweeds (up to 200,000 cells/100 g of algae) and also occur freely in sediments and coral rubble. These dinoflagellates produce the potent neurotoxins gambiertoxin and maitotoxin (see Chapter 10),

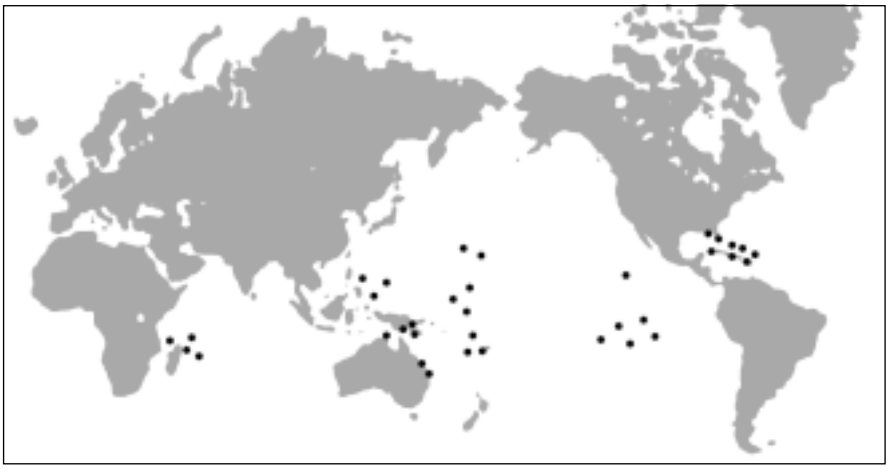

Figure 1.10
Global distribution of ciguatera fish poisoning (CFP).

which accumulate through the food chain, from small fish grazing on the coral reefs into the organs of bigger fish that feed on them (the principal toxin fraction in fish is ciguatoxin). While in a strict sense this is a completely natural phenomenon (Captain Cook suffered from this illness when visiting New Caledonia in 1774), from being a rare disease two centuries ago ciguatera now has reached epidemic proportions in French Polynesia. In the period 1960–1984 more than 24,000 patients were reported from this area, which is more than six times the average for the Pacific as a whole. Evidence is accumulating that reef disturbance by hurricanes, military and tourist developments, as well as coral bleaching (linked to global warming), are increasing the risk of ciguatera by increasing benthic substrate for dinoflagellate growth (Bagnis *et al*., 1985).

1.5 Stimulation of algal blooms by unusual climatological conditions

1.5.1 Toxic Pyrodinium bahamense blooms in the tropical Indo-West Pacific

At present the dinoflagellate *Pyrodinium bahamense* is confined to tropical, mangrove-fringed coastal waters of the Atlantic and Indo-West Pacific. A survey of fossil occurrences of its resting cyst *Polysphaeridinium zoharyi* (Fig. 1.11) (records go back to the Eocene, 50 million years ago) indicates a much wider range of distribution in the past. For example, in the Australasian region at present the dinoflagellate does not extend further south than Papua New Guinea, but in the Pleistocene it ranged as far south as Sydney Harbour (McMinn, 1989). There is genuine concern that, with an increased greenhouse effect and warming of the oceans, this species may return to Australian waters. In the tropical Atlantic, in areas such as Bahia Fosforescente in Puerto Rico and Oyster Bay in Jamaica, this species forms persistent luminescent blooms which are a major tourist attraction. Both plankton bloom material and oysters and mussels attached to mangrove roots in Bahia Fosforescente appeared at one time to be non-toxic (Hallegraeff; Oshima, unpublished data). The first harmful implications of *Pyrodinium* blooms became evident in 1972 in Papua New Guinea. Red-brown water discolorations coincided with the fatal food poisoning of three children and mouse bioassays on shellfish from a house in the affected village subsequently established *Pyrodinium bahamense* as a source of paralytic shellfish poisons (MacLean, 1977). Since then, toxic *Pyrodinium* blooms have apparently spread to Brunei and Sabah (1976), the central Philippines (1983), the northern Philippines (1987) and Indonesia (North Mollucas). MacLean (1989) presented strong circumstantial evidence for a coincidence between *Pyrodinium* blooms and El Niño-Southern Oscillation (ENSO) climatological events. El Niño is caused by an imbalance in atmospheric pressure and sea temperature between the eastern and western parts of the Pacific Ocean and results in a shoaling of the thermocline. The 1991–1994 ENSO event and recurrence of dinoflagellate blooms in the Philippines tend to substantiate these claims (Fig. 1.12).

Pyrodinium is a serious public health and economic problem for the tropical countries that are affected, as they depend heavily on seafoods for protein and have little prior experience in toxic dinoflagellate research. In the Philippines alone, this organism has now been responsible for more than 2,000 human illnesses and

100 fatalities resulting from the consumption of contaminated shellfish, as well as planktivorous fish such as sardines and anchovies. Most unexpectedly, during a *Pyrodinium* bloom in 1987 on the Pacific coast of Guatemala, 187 people had to be hospitalized and 26 died. In 1989 another bloom swept northward along the Pacific coast of Central America, again causing illness and death. The Guatemala populations are morphologically more similar to the Indo-West Pacific populations (sometimes distinguished as a separate variety *compressum)* than to the Caribbean morphospecies (var. *bahamense)* (Rosales-Loessener *et al*., 1989).

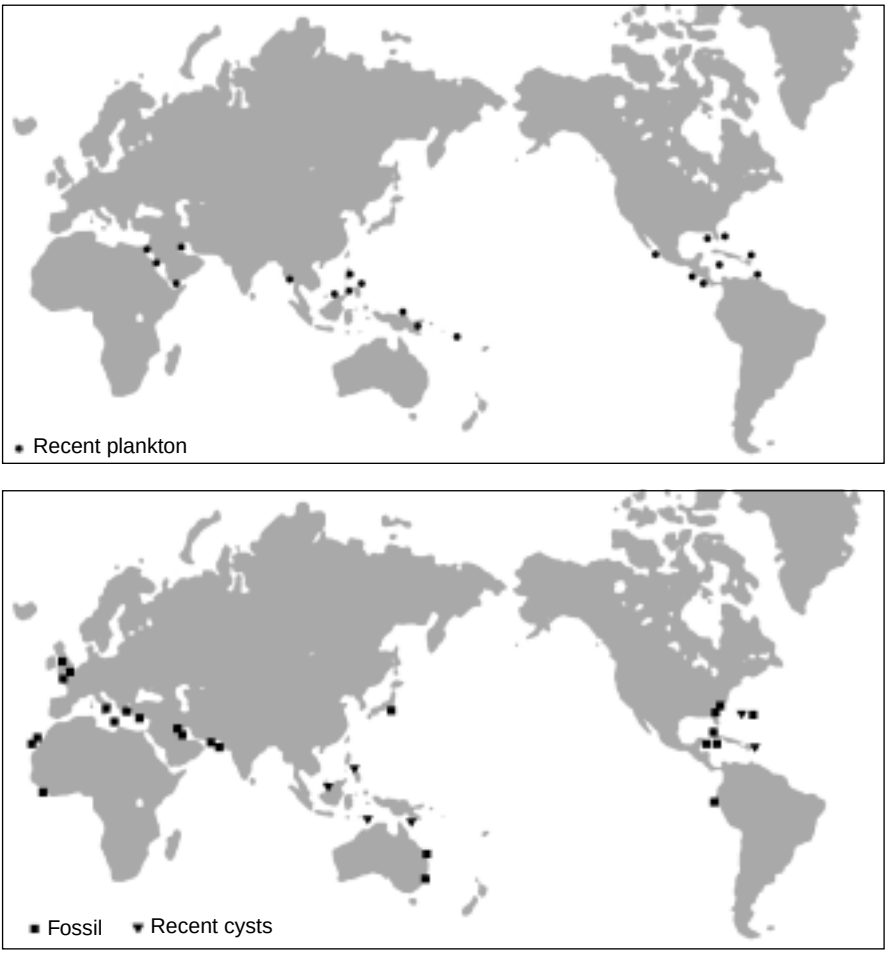

Figure 1.11
Global distribution of the tropical dinoflagellate *Pyrodinium bahamense* in recent plankton (above) and of the fossil cyst *Polysphaeridinium zoharyi* (below).
Source: Hallegraeff and MacLean (1989).

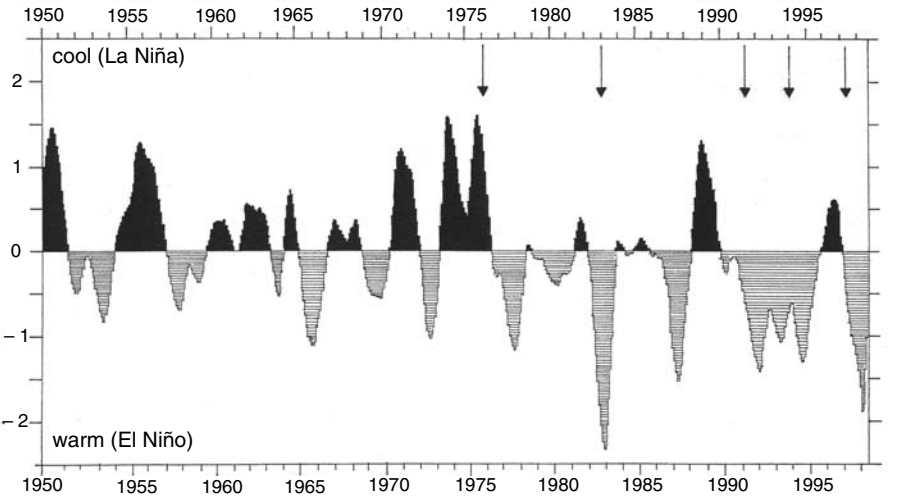

Figure 1.12
Relationship between *Pyrodinium bahamense* blooms (arrows) in the tropical Indo-West Pacific and El Niño–Southern Oscillation (ENSO) climatological events in 1970–1998. The graph shows mean surface temperature anomalies over the near-equatorial eastern Indian and western Pacific Oceans. Strong positive anomalies are indicative of ENSO events.
Source: Azanza and Taylor (2001)

1.5.2 Neurotoxic shellfish poisoning in Florida (USA) and New Zealand

Until recently, neurotoxic shellfish poisoning (NSP: Table 1.2), caused by polyether brevetoxins produced by the unarmoured dinoflagellate *Karenia brevis*, was considered to be endemic to the Gulf of Mexico and the east coast of Florida, where 'red tides' had been reported as early as 1844. An unusual feature of this organism is the formation by wave action of toxic aerosols that can lead to respiratory asthma-like symptoms in humans. In 1987 a major Florida bloom event was dispersed by the Gulf Stream northward into North Carolina waters, where it has since continued to be present (Tester *et al.*, 1991). Unexpectedly, in early 1993 more than 180 human shellfish poisonings were reported from New Zealand, caused by a number of species similar to *Karenia brevis*, now newly described as *K. bicuneiformis, K. brevisulcata, K. papilionacea, K. selliformis* (Jasperse, 1993; Haywood and Steidinger, in prep.). Most likely, this was a member of the hidden plankton flora (previously present in low concentrations), which developed into bloom proportions triggered by unusual climatological conditions (higher than usual rainfall, lower than usual temperature) coinciding with an El Niño event.

1.5.3 Fossil blooms of *Gymnodinium catenatum* in the Kattegat-Skagerrak

The present-day distribution of the paralytic shellfish poison-producing dinoflagellate *Gymnodinium catenatum* includes the Gulf of California, Gulf of Mexico, Venezuela, Argentina, Japan, Korea, China, the Philippines, Palau, Tasmania (Australia),

New Zealand, the Mediterranean and the Atlantic coast of Spain, Portugal and Morocco (Hallegraeff and Fraga, 1997). The microreticulate cysts of a closely related (apparently nontoxic) species *Gymnodinium nolleri* were present in unusually large amounts in pollen records from Kattegat sediments (Nordberg and Bergsten, 1988). A multi-disciplinary study (Dale and Nordberg, 1993) to reconstruct the prevailing paleoenvironment has suggested the following scenario: the migration of this organism into the area about 5000 B.P.; its establishment as part of the local plankton; a major blooming phase about 2000–500 B.P. of a magnitude that has not been seen since; and its disappearance during the 'Little Ice Age'.

1.6 Transport of dinoflagellate cysts in ballast water or during translocation of shellfish stocks

Cargo-vessel ballast water was first suggested as a vector in the dispersal of non-indigenous marine plankton some ninety years ago. However, in the 1980s the problem of ballast-water transport of plankton species gained considerable interest when evidence was brought forward that non-indigenous toxic dinoflagellate species had been introduced into sensitive aquaculture areas of Australian waters, with disastrous consequences for commercial shellfish farm operations (Hallegraeff and Bolch, 1992). While the planktonic stages of diatoms and dinoflagellates show only limited survival during the voyage in dark ballast tanks, their resistant resting spores are well suited to survive these conditions. One single ballast tank was thus estimated to contain more than 300 million toxic dinoflagellate cysts which could be germinated into confirmed toxic cultures. Paralytic shellfish poisoning was unknown in the Australian region until the 1980s when the first outbreaks appeared in the ports of Hobart (*Gymnodinium catenatum*), Melbourne (*Alexandrium catenella)* and Adelaide (*A. minutum)*. In Hobart, Tasmania, an examination of historical plankton samples and cyst surveys in dated sediment depth cores (McMinn *et al*., 1997) provided strong circumstantial evidence that the toxic dinoflagellate *G. catenatum* was introduced after 1973. Furthermore, in Melbourne and Adelaide, genetic fingerprinting using rRNA sequencing provided circumstantial evidence for the genetic affinities between Australian and Japanese strains of *A. catenella* and Australian and European strains of *A. minutum* (Scholin *et al*., 1993; de Salas *et al*., 2000). The toxic dinoflagellate *Pfiesteria* has been confirmed from ballast water entering North America from Europe (P. Rublee, unpublished).

The evidence of ballast-water transfer of marine organisms other than microscopic algae is considerable and includes species of seaweeds, fish, crustaceans, polychaete worms, starfish and molluscs (Carlton, 1985). As of 1 November 1991, the International Maritime Organization (IMO) has ratified the introduction of voluntary guidelines for ballast-water handling procedures by bulk-cargo vessels. These measures aim to reduce the risk of harmful introductions by encouraging a range of practices such as reballasting at sea (only feasible for vessels up to 40,000 dead weight tonnage), ballasting in deep water and disposal of ballast-tank sediments away from sensitive aquaculture or marine park areas. The most effective measure to prevent the spreading of dinoflagellate cysts via ships' ballast water would be to avoid ballasting during toxic dinoflagellate blooms in ports. Other options using heat, electrical shock or chemical treatment (chlorine, hydrogen peroxide) of ballast

water, either in hold or in onshore facilities, have also been explored (Hallegraeff, 1998).

Another vector for the dispersal of algae (especially their resting cysts) is with the translocation of shellfish stocks from one area to another. The faeces and digestive tracts of bivalves can be loaded with viable dinoflagellate cells and sometimes can also contain resistant resting cysts (Scarratt *et al*., 1993; Schwinghamer *et al*., 1994). The Japanese seaweeds *Sargassum muticum* (United Kingdom, Netherlands, Norway), *Undaria pinnatifida* and *Laminaria japonica* (Mediterranean) thus are thought to have been introduced into European waters via sporophyte stages contained with introduced Japanese oyster spat.

1.7 Conclusions

Whether the apparent global increase in harmful algal blooms represents a real increase is a question that we will probably not be able to answer conclusively for some time to come. There is no doubt that the growing interest in utilizing coastal waters for aquaculture is leading to greater awareness of toxic algal species. What we are faced with today in the field of harmful algal bloom research is that the effects on public health and the economic impact of harmful algal blooms are showing signs of a truly global 'epidemic' and we should start to respond to this problem. In countries that pride themselves on their disease- and pollution-free status for aquaculture, every effort should be made to quarantine sensitive aquaculture areas against the unintentional introduction of non-indigenous harmful algal species. Furthermore, no aquaculture industry can avoid having to monitor for an increasing number of harmful algal species in the water column and for an increasing number of algal toxins in seafood products.

Most importantly, those responsible for management decisions on pollutant loadings of coastal waters (including decisions on agricultural and deforestation activities in catchment areas) should be made aware that one probable outcome of increased nutrient loading will be an increase in harmful algal blooms. Finally, global climate change studies (El Niño, greenhouse, ozone depletion) need to consider possible impacts on algal-bloom events. A number of new international programmes have been created to study and manage harmful algal blooms and their links to environmental changes in a manner consistent with the global nature of the phenomena involved (see Appendix B). It is hoped that this *Manual on Harmful Marine Microalgae* will facilitate these international efforts.

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2

Sampling techniques and strategies for coastal phytoplankton blooms

P. J. S. Franks and B. A. Keafer

The primary goal of sampling for coastal toxic phytoplankton is to gain some predictive ability concerning the initiation of local outbreaks through an understanding of the processes controlling the cell distributions. A fundamental question is whether the bloom formed *in situ*, or was advected to the region. In regions of weak physical forcing – embayments or lagoons with weak tidal flushing – it is likely that the bloom formed *in situ*. Prediction of toxic outbreaks in this case would depend on a thorough knowledge of the physiology, life cycle and behaviour of the phytoplankter, and the relationship of these responses to environmental forcings such as light or nutrient loading. Most coastal areas, however, are strongly influenced by physical forcings, making identification of transport mechanisms of paramount importance in predicting the timing and location of toxic outbreaks. This chapter describes sampling equipment and strategies for identifying the local causes of toxic phytoplankton blooms and suggests techniques that may aid in gaining some predictive capability for harmful algal blooms in coastal regions.

The fundamental key to developing early warning or predictive capability for toxic outbreaks is a well-designed field-sampling programme. Some field programmes are research-oriented, focusing on the underlying causes and dynamics of the blooms while others are designed for monitoring purposes to determine the relative abundance of toxic species present in the local waters. Whether the sampling effort is research-oriented or part of a monitoring programme, designing a field programme includes consideration of both spatial and temporal domains, with one basic point: the sampling must begin before the bloom occurs. There is no way to accurately identify the source population(s) or proximate cause of a bloom after it has begun, unless historical data are available. This point will constrain all other aspects of the sampling scheme, including spatial arrangement of sampling stations, and timing of cruises. These points are expanded upon below, following a brief description of the equipment commonly used to sample coastal phytoplankton.

2.1 Sampling equipment

Secondary in importance to the timing and spatial arrangement of stations is the equipment used to perform the sampling. This equipment can range from the very simple, e.g. buckets and nets, to relatively complex, e.g. hose-pumping systems or rosette samplers. Much of the equipment described here is more thoroughly presented in the UNESCO *Phytoplankton Manual* (Sournia, 1978). In spite of its age, the information in that volume is still pertinent today.

2.1.1 Buckets and nets

The simplest pieces of equipment for sampling coastal phytoplankton are buckets and nets that can easily scoop surface water from a dock, shore, or from the side of a boat. If quantitative estimates are needed, a subsample of known volume should be removed from the bucket for immediate examination or preserved for later analysis of the toxic cells present. Nets are commonly used to concentrate phytoplankton, especially when toxic cells are not a major component of the community. The main consideration when using a net or sieve is the mesh size relative to the species being sampled. Too large a mesh will eliminate small species. Nets are very useful for gathering qualitative presence/absence information, but because relatively small-mesh phytoplankton nets (e.g. 20 μm) typically clog rapidly when towed, they should never be used for quantitative estimation of cell numbers. Qualitative phytoplankton sampling using nets and near real-time microscopic observations has complemented several state shellfish-monitoring programmes in the USA. Those monitoring programmes rely on trained citizenry in affected coastal areas and have been successful in identifying the presence of toxic species prior to outbreaks of PSP and ASP (Sherwood Hall, pers. comm., 2000). If the net is not towed, but rather a known volume of seawater is pumped through the net or poured through mesh fixed to the bottom of a cylinder, a concentrated, quantitative sample can be collected. For more precise quantitative work, it must be ensured that the cells of interest do not rupture during the sieving process and that care is taken to wash all the cells off the net or sieve. Relatively robust armoured dinoflagellate cells, (e.g. *Alexandrium*) have been collected in this manner for both quantitative toxin measurements from net samples (Poulton, 2000) and quantitative cell counts from sieved samples (Franks and Anderson, 1992*a*, Anderson, unpublished data). Nets cannot be used to sample the vertical distributions of cells, unless the samples are obtained by other means (i.e. by tube samplers, bottles, or pumps – see below) then passed through the net. Other aspects of net sampling are well covered by Tangen (1978).

2.1.2 Tube samplers

Second to the net in simplicity are tube and segmented-tube samplers. The former (Franks and Anderson, 1989; Fig. 2.1A) acquires integrated samples of shallow (<5 m deep) water columns, or integrated samples from the surface waters of deeper regions. It consists of a 2–3 m length of PVC pipe, fitted with a rubber stopper attached to a cord threaded through the pipe to a handle at the top. A short foot at the bottom keeps the sampler from being pushed into the benthos. The sampler is slid vertically into the water until the foot rests on the bottom. Once the depth to the bottom is known, the tube is raised and moved to a new location nearby, and lowered until the foot is just off the bottom. This procedure prevents the water column samples from being contaminated with resuspended material. The cord is then pulled to seal the tube with the cork. The tube is emptied by pouring from the top, as pouring from the bottom causes spillage around the rubber stopper. The integrated vertical sample can be poured into a carboy or bucket for further subsampling (see below). The advantages of this type of sampler are: (a) low cost; (b) ease of construction; (c) one sample integrates any vertical heterogeneity of the organism; and (d) ease of deployment. The disadvantages include: (a) no vertical resolution; (b) relatively small volume; and (c) no real-time (i.e. instantaneous) vertical information of hydrographic parameters.

In shallow areas and relatively calm seas, the segmented tube sampler described by Lindahl (1986) and Sutherland *et al*. (1992) may be the most appropriate sampling device. This low-cost sampler consists of lengths of PVC pipe or garden hose, linked with valves and easily separated connectors (Fig. 2.1B). The length of a section determines the vertical resolution of sampling, typically 1–3 m, with a total length of up to 20 m. The sampler is slowly lowered with all valves open until the hose is filled. The top valve is then closed and the hose raised until the next valve down can be closed. The upper section can now be removed and its water drained. This procedure can be repeated until the whole length of hose has been raised. Hydrostatic forces within the hose will hold the water within the hose while it is being raised. Thus a small-volume vertical profile of the water column is obtained. The advantages of this sampler are similar to those of the tube sampler described above, although vertical resolution is obtained. The disadvantages are: (a) small volume; (b) no real-time vertical information; and (c) smearing of vertical structure within the pipe during filling due to its narrow diameter.

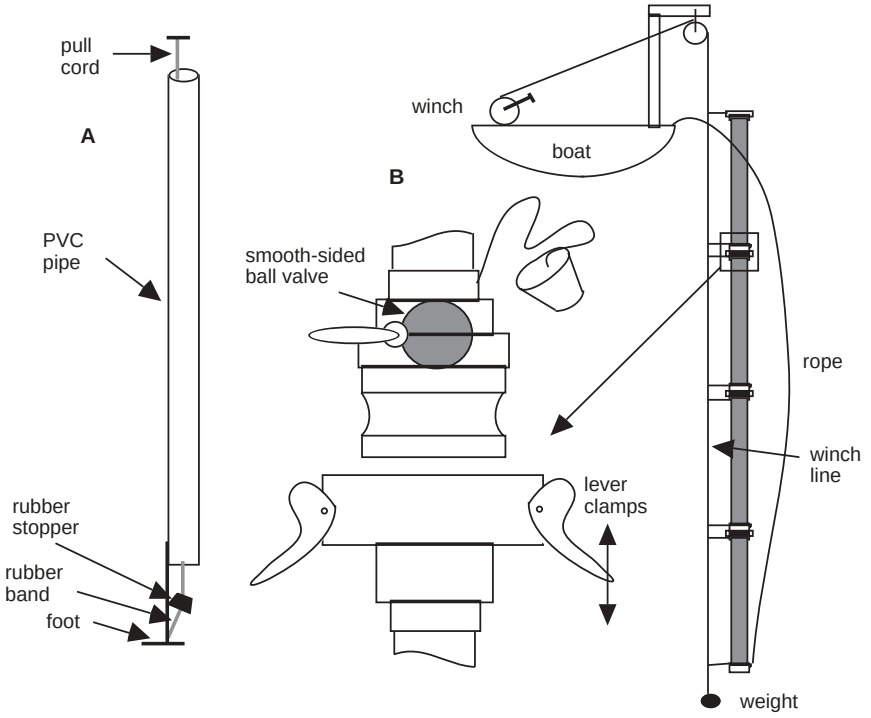

Figure 2.1
A, tube sampler; B, segmented-tube sampler and detail of connector.
Source: after Sutherland *et al*. (1992).

2.1.3 Bottles and rosettes

Most studies that examined the distribution of harmful phytoplankton species relied on bottles for obtaining samples. Bottles are relatively inexpensive, very robust, can be deployed from almost any vessel and give an accurate quantitative representation

of species densities within the depth sampled. Bottles are especially recommended for use in heavy seas, as they are easy to deploy and have no components to tangle or kink as with pumping systems. A good description of the types of bottles and their use may be found in Venrick (1978).

Niskin bottles are the type most commonly used today. They are available in various sizes ranging from about 1–30 l to accommodate the volume requirements of most sampling programmes. Smaller bottles may be adequate when the abundance of a target HAB species is high, whereas larger bottles are necessary when the target species are relatively rare (e.g. sampling prior to a bloom event to detect source populations of vegetative cells or even resuspended cysts). Larger bottles also allow replicate cell samples or associated water and/or cell properties to be measured from the same water bottle (nutrient, toxin samples, etc.), but are more difficult to handle unless they are deployed from a rosette.

Commonly, several bottles are suspended from a line or wire in the open position. When the bottles reach the desired depths, they are tripped closed with 'messengers' slid down the line. Alternatively, bottles can be arranged in a rosette formation usually configured with a conductivity–temperature–depth (CTD) sensor (see hydrographic samplers). Either method gives adequate vertical spatial coverage for most studies if enough bottles are available, otherwise repeated casts are necessary. Some studies (e.g. Legovic *et al*., 1991) have even used bottles deployed by divers to obtain samples from visible strata of dinoflagellate accumulations to make sure that the layer of interest was sampled. With the CTD, hydrographic data can be acquired and displayed in real-time during the downcast. During the subsequent upcast, the bottles can be electronically closed at predetermined depths and/or at interesting features (e.g. within the chlorophyll *a* maximum layer). The data files generated include a record of the precise depths and associated hydrographic data when the bottle is closed.

2.1.4 Pumping systems

Most coastal areas are dynamically complicated, requiring detailed coverage of both biology and hydrography. While bottle samplers are usually adequate, they are nevertheless somewhat limiting due to their small volume and inability to provide continuous vertical profiles. If more detailed vertical profiles are required, for example if the phytoplankton are aggregated into a thin layer which is difficult to sample with a bottle, a pump-profiling system may be an appropriate tool. These systems have the advantages of: (a) high volume, permitting sampling of a number of variables; (b) continuous vertical profiles; and (c) real-time observations of vertical features. The disadvantages are: (a) relatively high cost (generally over US$300 depending on the sophistication of the system); (b) more difficult to deploy than the samplers described above; and (c) require ac or dc power source for the pump and associated instruments.

The pump-profiling system at its simplest is a pump and one or more lengths of hose. Aspects of the system design which must be evaluated include the position of the pump (at surface or at depth), the pump head (the vertical distance the pump can push water), the insertion of various flow regulators and a bubble trap, and the inclusion of subsidiary sampling devices such as fluorometers, autoanalyzers, etc. (Fig. 2.2). These aspects are well described by Beers (1978), Franks and Anderson (1989) and Powlik *et al*. (1991). Pumps can range from a peristaltic pump (Voltolina,

1993) or a centrifugal diaphragm pump (Taggart and Leggett, 1984) to a watertight submersible pump (Miller and Judkins, 1981). Swimming-pool pumps and sump pumps are relatively inexpensive, waterproof, and available through most hardware or plumbing stores.

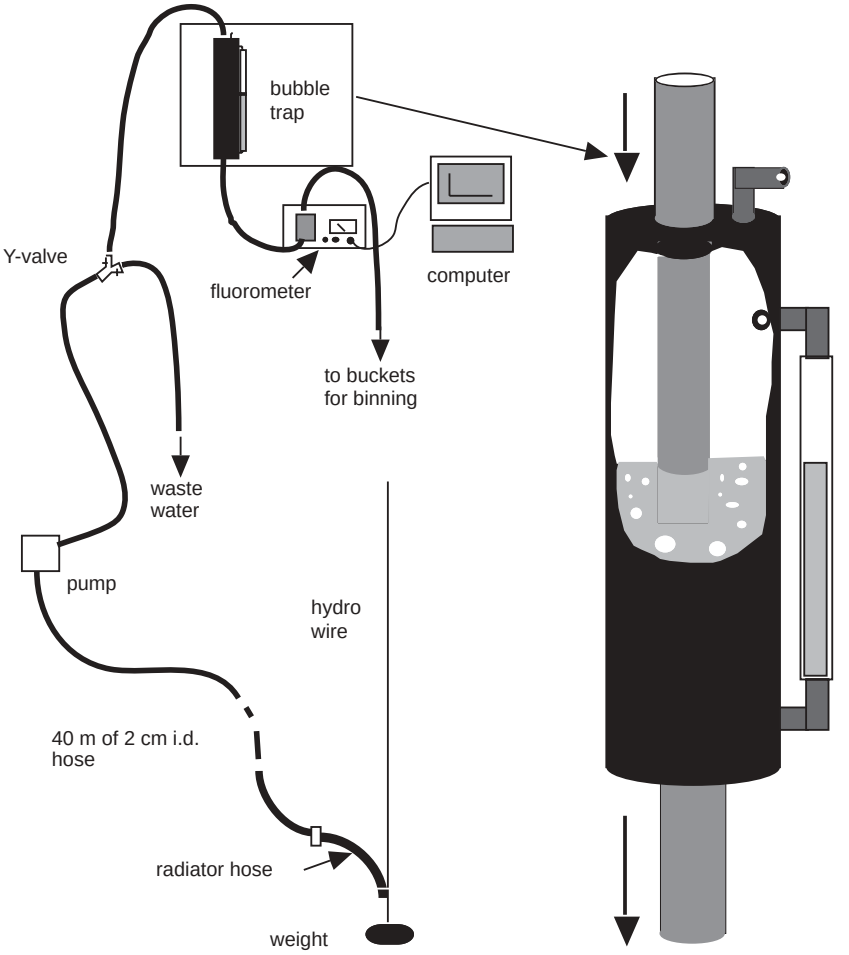

Figure 2.2
Example of a hose-pumping system and detail of the bubble trap.
Source: after Franks and Anderson (1989).

Many of the pumping systems described in the literature have been designed for the sampling of zooplankton and therefore pump large volumes of water through a mesh of some sort to concentrate the samples. As phytoplankton are so abundant, this will usually not be a problem and sample volumes can be smaller and flow rates lower. Systems designed for sampling phytoplankton typically have a junction beyond the pump to allow splitting of the pump discharge: the bulk will be discarded

overboard, while a relatively small stream will be passed through instruments or used for integration or subsampling (Franks and Anderson, 1989).

The protocol for sampling with the pump profiling system is complicated by the transit time of the water through the hose. Water that is being pumped on deck was obtained from the depth the hose inlet occupied some time earlier. To correct for this requires measurement of the transit time of water through the hose using coloured dye or a flow-through fluorometer to detect a spike of chlorophyll introduced at the hose inlet (e.g. Franks and Anderson, 1989). The calculated transit time should be at the first appearance of the dye at the end of the hose: smearing within the hose will cause an initial spike to be spread over a considerable distance within the hose.

Bubbles within the hose can be a serious problem with certain instruments such as fluorometers and autoanalyzers. To mitigate the problem, a bubble trap can be included before the water reaches the instruments (Fig. 2.2). One design consists of a 1 m length of 10 cm internal diameter acrylic pipe, fitted with stoppers and hose connectors at the top and bottom. A small chimney tube at the top allows air to escape. A length of clear tubing joined to the top and bottom with right-angled connectors allows the water level to be seen within the pipe. A 20 cm length of hose extends from the top hose connector into the bubble trap. The level of water within the bubble trap should always be kept above the level of this hose so that no more bubbles are created. The bubble trap itself should be located at the highest point of the pumping system, so that all bubbles within the system may escape. The height of the bubble trap will largely be determined by the head of the pump and the geometry of the ship.

From the bubble trap, the flow feeds by gravity into the attached instruments and back to the deck. The water may be collected in carboys or buckets to obtain samples integrated over any desired depth interval as the hose is raised or lowered. This is known as 'binning'. For certain analyses (e.g. productivity or chlorophyll) the sample containers should be opaque or acid-washed, although a sea-water rinse is sufficient for species counts.

Subsampling from the bins depends on the information needed. For example, Franks and Anderson (1989) sieved 1 l from each 5 m depth bin through 20 μm Nitex mesh (epoxied onto a cylinder of 8 cm PVC pipe) and washed the retained cells into sample tubes containing 5% formalin for cell counts. An additional 500 ml was filtered through GF/A filters that were used for chlorophyll analyses. The filtrate was frozen for nutrient analyses.

Regardless of which method is chosen for collection of the cells, preservatives are required if the samples are to be stored. Common phytoplankton preservatives are Lugol's solution and the aldehydes, both glutaraldehyde (~1%) and formalin (~5%). The reader is referred to Sournia (1978) for a good discussion of the advantages and disadvantages of various preservatives. More recently, species-specific molecular probes, such as antibodies and oligonucletides, are being used to fluorescently-label individual species in mixed assemblages (Anderson, 1995). In general, formalin (5%) works well to preserve cell surfaces for antibody labelling. Glutaraldehyde should be avoided due to higher background autofluorescence. Chlorophyll autofluorescence within the cell will interfere with the visualization of fluorescent labels that bind to rRNA targets within the cell. To alleviate this problem, methanol and ethanol can be used alone or in conjunction with formalin to preserve the cells, while also extracting the chlorophyll (Anderson *et al.*, 1999; Miller and Scholin, 2000). Thus, choice of preservative used in the field will not only depend on how

well the target cells (e.g. armoured *v.* unarmoured dinoflagellates) can be preserved but also by the method used to detect and enumerate the cells. Chapter 4 of this *Manual* gives further information on counting of phytoplankton.

2.1.5 Hydrographic samplers

An essential part of any sampling programme is the acquisition of hydrographic data, specifically temperature and salinity versus depth. This has been stressed for decades (Seliger and Holligan, 1985) and is repeated here: coincident biological and physical data are necessary for the identification of transport mechanisms for noxious phytoplankton blooms. In regions of strong freshwater input or thermal forcing, such basic instruments as reversing thermometers and a hand-held refractometer may suffice. The refractometer has an accuracy of about 0.2 psu (practical salinity units; 1 psu = 1 ppt), which is reasonable for most coastal areas with strong freshwater influence. If sophisticated instruments are not available, temperature measurements using a thermometer and salinity samples measured using a refractometer should be taken at least at 0.5 m intervals. For more accurate salinity measurements, samples can be collected and analysed in the laboratory by a salinometer. These measurements can be made on water exiting the hose if a pumping system is used. Obviously, vertical resolution will be compromised if a pumping system is not available, as bottle sampling is not conducive to high vertical-resolution sampling.

In dynamic regions with weaker variations in properties, a small, self-contained CTD is an invaluable tool. While such instruments may be expensive for a small laboratory, the investment may be worthwhile in the excellent quality of data returned, and the ease of deployment and data reduction. Inexpensive battery-operated temperature/conductivity probes, such as those manufactured by InterOcean Systems, Inc. (3540 Aero Ct., San Diego, CA, USA 92123, http://www.interoceansystems.com/) can be lowered to learn details of water column structure. A relatively inexpensive CTD profiler that we recommend for coastal work is the 'Sea Cat Profiler' (about US$7,000 base model; Sea Bird Electronics, 1808-136th Pl. NE, Bellevue, WA, USA 98005, http://www.seabird.com/). This small instrument can be easily transported to the study site and is practically indestructible and foolproof. It stores data internally during a cast, requiring only a magnetic switch to be turned on to record the cast. No extra electrical wires are required over the side of the ship. Mutiple casts can be recorded and the data subsequently transferred to a laptop computer using the programs supplied with the CTD. This CTD can be hung from a hydrowire, mounted below the hose inlet of a pumping system, alongside a water bottle, or in a rosette array. An additional advantage of many CTD profilers is that they can be expanded to include *in situ* fluorometers, transmissometers, light meters, O2 sensors, etc. These instrument packages are easily deployed even in fairly rough seas, when use of a pumping system may be impossible. Because deployment is relatively easy, CTDs also allow dense sampling of a variety of fields.

More sophisticated CTD/rosette samplers are available from several manufacturers at much higher cost (about US$25,000). The main advantage with these systems is greater accuracy and faster response of the sensors, much higher frequency of sampling, and more ports for redundant or auxillary sensors. The resulting vertical profiles contain fewer anomalous data points, e.g. salinity spikes, especially when the CTD is deployed through the pycnocline where water properties are rapidly

changing. Typically these systems require a 'slip-ring' winch rigged with conducting wire for power and data transmission, but some CTD models are also available with self-contained power and sufficient memory to internally record the high-resolution data as well as to close sampling bottles at preprogrammed depths. Another more sophisticated system might include a flow through thermosalinograph (available through InterOcean Systems, Inc.) integrated into a hose-pumping system on deck. This instrument may also be interfaced to a computer, allowing rapid data acquisition and storage. Similarly, a flow-through system integrated with a seawater intake on the vessel is useful for underway sampling to detect surface hydrographic features (fronts) that may contain toxic species.

2.1.6 Satellite- or radio-tracked drifters

Recent technology has allowed drift bottles to evolve into relatively inexpensive yet sophisticated drifters that can be tracked either by satellite (ARGOS) or Global Positioning System (GPS) signals. They can be easily deployed within water masses that contain HAB species to establish transport pathways and estimate rates of transport. An account must be established with ARGOS for the tracking charges, where the account owner receives the positions of the drifter along with optional sea-surface temperature data (if the sensor is on the drifter) via e-mail. Several companies manufacture the drifters using two main designs. The Davis type (about US$1,000–1,500; Technocean Inc., http://www.technocean.com/) is designed with four vertical underwater 'sails' that track the top 1 m of water. The 'holey-sock' design (Sea-Gear Corporation, http://www.sea-gear.net/; Bridport Industries Ltd., Canada) is essentially a cylindrical cloth (attached to supports for rigidity) that can be drogued near the surface or extended to depth. These drifters are usually considered expendable, as the cost of relocation and pick-up is usually greater than the cost of a new drifter. However, some models are available with optional GPS transmitters (about US$2,500, Brightwaters Inc., http://www.brightwaters.com/) that allow real-time tracking and recovery using the ship's GPS system. These are particularly useful for diel studies of phytoplankton dynamics (for vertical migration studies) where the drifter is used to follow a patch while hydrographic data and phytoplankton samples are collected nearby.

The quality and durability of such instruments is of paramount importance: nothing is more frustrating than trying to collect data with unreliable instruments. Always try to test an instrument in the field before purchase, and talk to others who have used the instrument. One final word of advice on sampling equipment: always plan for the worst. Bring spares of all pieces of equipment: hose, pumps, valves, connectors, hose clamps, etc. A supply of duct tape is a necessity. Always make contingency plans if any aspect of the sampling programme should fail at sea, e.g. if the pump loses its prime, the computer fails, or the fluorometer breaks. Planning ahead for such emergencies will help to make the best out of a bad situation and may prevent the waste of time and money.

2.2 Physical forcings

A feature of noxious blooms that has been noted for decades is that the blooms are often associated with distinct water masses (Mead, 1898; Slobodkin, 1953; Kierstead and Slobodkin, 1953; Conover, 1954; Ryther, 1955; Cullen *et al*., 1982; Carreto *et al*., 1986; Fraga *et al*., 1988; Dundas *et al*., 1989; Franks and Anderson, 1992*a*; Townsend *et al.*, 2001). This attribute is central to the identification of transport mechanisms and the prediction of local outbreaks. Once the association between toxic phytoplankton and water mass has been identified, sampling schemes can concentrate on elucidating the processes influencing the timing, size, location and direction of travel of the water mass (Tyler and Seliger, 1978; Dundas *et al*., 1989; Franks and Anderson, 1992*a*, 1992*b*). In this section we discuss physical mechanisms known to be important in the regional transport of toxic phytoplankton in coastal regions, and optimal temporal and spatial arrangement of sampling to identify the important physical dynamics.

The most important transport mechanisms in coastal regions are those forced by buoyancy (e.g. freshwater outflows), wind, tides and topography (Franks, 1992; Fig. 2.3). It is quite likely that several of these forcings will be operating at any one time. However, usually only one mechanism will be responsible for the initial formation of the water mass that supports the toxic algae. Other forcings may come into play in the transport of this water mass. It is important to distinguish between the formation and the transport of the water mass for the prediction of local outbreaks.

2.2.1 Buoyant plumes

Central to the understanding of coastal currents is the Coriolis effect, which causes a deflection of currents to the right in the Northern Hemisphere (to the left in the Southern Hemisphere). Many alongshore flows are created as a result of a balance between a pressure gradient (warm, fresh light water that tends to flow over cold, salty, dense water) and the Coriolis effect. A buoyant plume formed by freshwater discharge from an estuary will deflect to the right in the Northern Hemisphere upon exiting the estuary and flow along the coast (Beardsley and Hart, 1978; Woods and Beardsley, 1988; Chao, 1988*a*) if no other forces are acting on it. With no Coriolis effect, the freshwater would spread radially from the estuary mouth. The deflection of the current to the right creates a relatively narrow hydrographic feature which is trapped at the coast and propagates along the coast at a speed determined by the volume of freshwater discharge and external forcing such as remotely forced alongshore flows (Beardsley and Hart, 1978; Woods and Beardsley, 1988), wind (Chao, 1988*b*), tides, or topography (Butman, 1976; Franks and Anderson, 1992*a*).

2.2.2 Wind-forced flows

Wind-forced flows also deflect to the right in the Northern Hemisphere (left in the Southern Hemisphere). This, combined with the constraint that the water does not flow through the coast, leads to the strong along- and across-shelf flows created by coastal winds. The phenomena of wind-driven upwelling and downwelling are well documented (Richards, 1981). While these flows are typically visualized in a cross-shelf plane, it is often forgotten that any across-shelf motion is accompanied by strong along-shore flows. A poleward wind blowing along the eastern coast of a land mass will generate an offshore flow of water (Fig. 2.4A). This offshore surface flow

must be balanced by an onshore flow of deeper water, which creates the upwelling front. At the same time, however, strong alongshore flows are generated, predominantly in the direction of the wind. A buoyant plume propagating along the coast would be forced offshore by the upwelling, and backwards against its natural direction of propagation by the wind (Fig. 2.4C). On the other hand, an equatorward wind along the same coast would force surface waters into the coast (downwelling), generating a strong flow of water in the direction of the wind along the coast (Fig. 2.4B). The same buoyant plume caught in this wind-forced flow would be pushed against the coast, and accelerated in its natural direction of propagation (Fig. 2.4D). It was

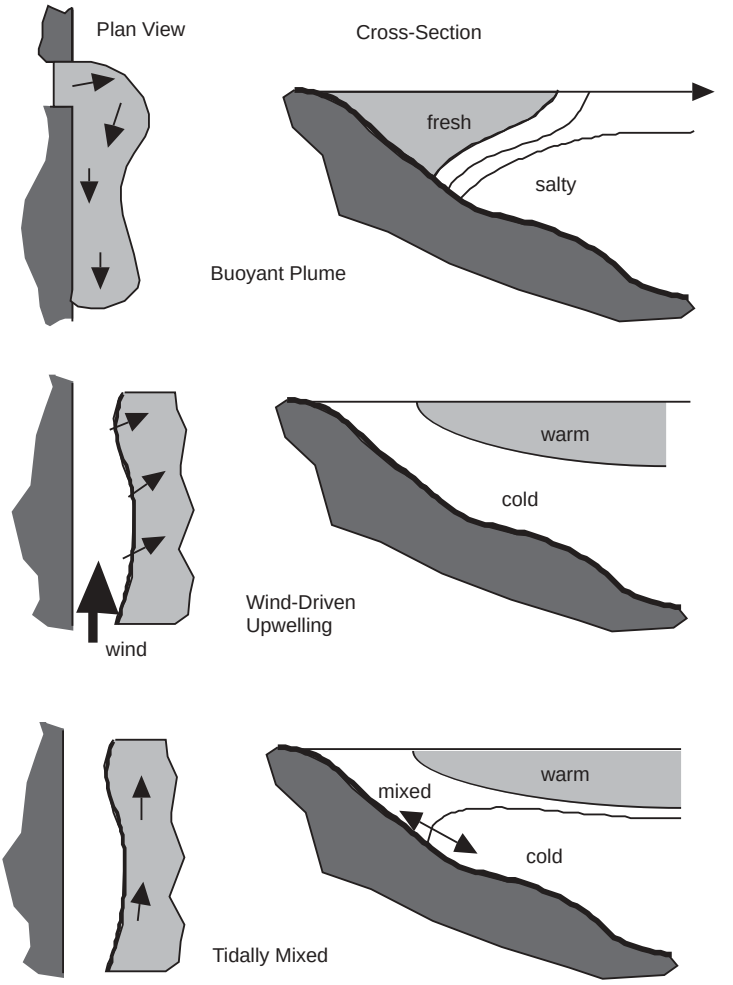

Figure 2.3
Plan and cross-sectional (cross-shelf) schematic views of a buoyant plume (above), wind-driven upwelling (centre), and a tidal front (below). Dark grey regions represent land, light grey shades indicate less-dense water.

found that this combination of buoyant plume and wind-forced dynamics explained the timing and location of PSP toxicity along the coast of the western Gulf of Maine (Franks and Anderson, 1992*a*, 1992*b*).

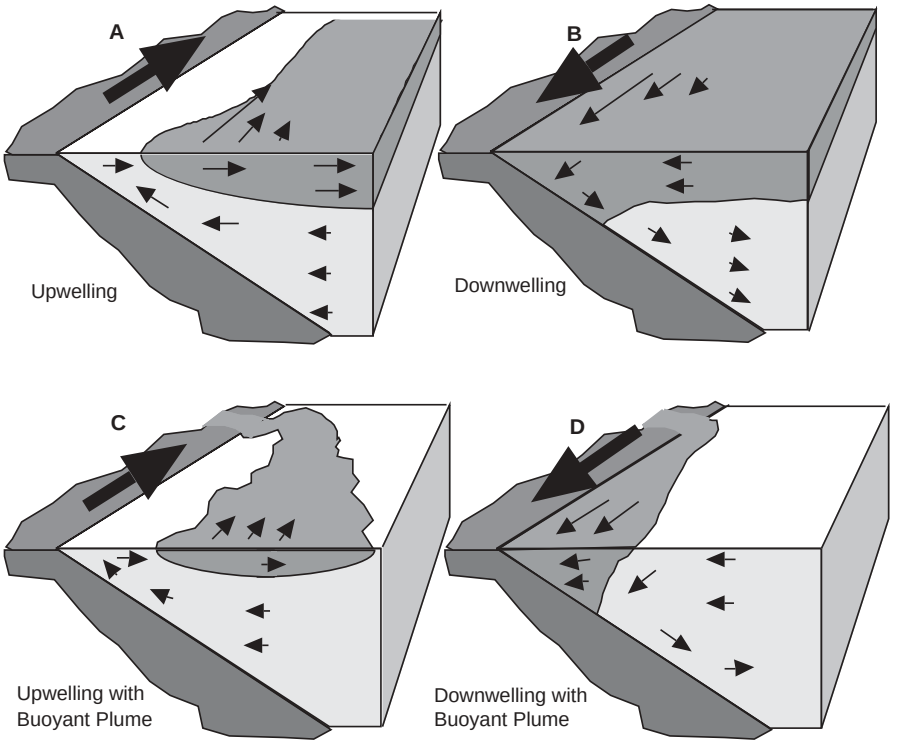

Figure 2.4
A, three-dimensional schematic representation of currents and water masses generated by wind-driven upwelling; B, currents and water masses generated by wind-driven downwelling; C, buoyant plume forced offshore by wind-driven upwelling; D, buoyant plume forced against the coast by wind-driven downwelling. Heavy arrows indicate wind direction, small arrows indicate water currents. Darker water masses are less dense (either warmer or less salty).

2.2.3 Tidal currents

Coastal tidal currents may transport material both parallel and perpendicular to the shoreline. While tides give particle displacements which are approximately elliptical in the horizontal (when seen from above), the sloping bottom and irregular topography in coastal regions can lead to residual tidal currents that produce a net alongshore transport of material (Greenberg, 1979; Pingree and Maddock, 1985; Geyer, 1993). This transport will vary in strength depending on the lunar cycle. Tides also interact with buoyant plumes and wind-forced flows in that they advect hydrographic

features back and forth. Because of this, a sampling station at a fixed geographic location may be in quite different water masses over the course of a tidal cycle.

Energetic tides in shallow waters may cause complete homogenization of the water column. In estuaries, homogenization can occur during spring tides, with water column stratification reestablished during the weaker, intervening neap tides (Haas, 1977). Mixing and homogenization may be an important factor for initiation of blooms from deeper phytoplankton populations or from benthic cysts (Haas *et al*., 1981). However, strong mixing may also terminate blooms by disruption of established surface populations. In coastal waters, homogenization may occur in shallow inshore areas, while deeper offshore waters remain stratified. The boundary between the mixed and the stratified water masses is often very sharp and is termed a tidal front (Bowman *et al*., 1986). Tidal fronts have been shown to be the site of enhanced dinoflagellate populations around the British Isles (Pingree *et al*., 1975, 1979), the Patagonian Shelf (Carreto *et al*., 1986), and may be the formation sites for noxious blooms in other regions of the world (Yentsch *et al*., 1986).

2.2.4 Topographic features

The influence of sharp topographic features, such as capes and islands, on coastal flows is not well understood. Some studies have examined the separation of buoyant plumes from the coast at capes (Butman, 1976; Bormans and Garrett, 1989; Franks and Anderson, 1992*a*, 1992*b*), while others have examined the influence of capes on wind-forced flows (Suginohara, 1974; Crepon *et al*., 1984). Pingree and Maddock (1979) and Wolanski *et al*. (1984) have examined the flows generated in the lee of an island embedded in a larger-scale flow. In general, the topographic feature may cause separation of the flow from the land, with the consequent formation of eddies and upwelling cells. If the toxic cells are carried in the coastal flow, this separation mechanism may shield the downstream side of the cape or island from toxicity (Franks and Anderson, 1992*a*, 1992*b*). On the other hand, the upwelling created by the eddies may stimulate phytoplankton growth, or create accumulation zones in which noxious populations may grow and eventually be advected downstream.

2.3 Sampling schemes

Given the variety and complexity of physical forcings in coastal oceans, a critical requirement of any sampling programme is that sampling must be done at more than one location. It is surprising how often this simple rule is broken, almost always with dire consequences. Even detailed hydrographic and biological samples taken frequently at a single point cannot tell you the direction of water motion or the scale of hydrographic variability. This is because *in situ* changes in properties cannot be distinguished from horizontal advection of gradients of properties. Any attempt to perform a mass balance in a coastal region will almost certainly conclude that the majority of changes in properties at a single station can be accounted for by horizontal advection. Any sampling programme must include a minimum of two, and preferably several more, stations in the across-shelf direction because across-shelf gradients in hydrographic properties give a great deal of information about the along-shelf flows. As mentioned above, horizontal pressure gradients in one direction lead to currents in the perpendicular direction due to the Coriolis effect

(the geostrophic balance). Thus a sampling scheme must be able to resolve sloping isopycnals in the across-shelf direction, which can give information on the direction of water motion.

The spacing between stations in the across-shelf plane is determined by the across-shelf scale of the hydrographic features that dominate the coastal region (Fig. 2.4). A good first estimate of this scale is the internal Rossby radius of deformation, R_i, often simply referred to as the Rossby radius (see Franks, 1992, for a thorough explanation of this scale, and its relation to a variety of fronts). The Rossby radius can be thought of as the radius of curvature of a current under the influence of the Coriolis effect and a pressure gradient. It is a natural scale for the curvature or width of wind-driven, topographic and buoyant plume fronts.

The Rossby radius, R_i, is calculated using the density difference between the surface and deep water masses, the thicknesses of the two water masses, the Coriolis frequency and gravity. The Coriolis frequency, f (units: s^{-1}), is calculated from the latitude, ϕ, and day length, and given by:

$$f = \frac{4\pi}{86400}\sin\phi .$$

If the surface waters have density ρ_s (units: kg m^{-3}) and thickness h_s (units: m), while the deep waters have density ρ_d and thickness h_d, and the acceleration due to gravity is g (~10 m s^{-2}), then the Rossby radius, R_i (units: m), is

$$R_i = \frac{\sqrt{g\dfrac{\rho_d - \rho_s}{\rho_d}\dfrac{h_s h_d}{h_s + h_d}}}{f} .$$

This radius is usually between 5 km and 20 km in coastal regions. As R_i gives the natural scale of horizontal variation for many hydrographic features, sampling should always be on scales smaller than R_i in order to resolve the natural variability.

The number of stations in the across-shelf direction will usually be determined by factors other than the hydrography, for instance the shelf width, the ability of the vessel to sample far offshore, available manpower, etc. A good rule to follow, however, is that samples should be taken out to at least $4R_i$ to $6R_i$ offshore, giving a transect of at least five stations. If real-time data are available to detect fronts where the change in temperature and/or salinity is greatest, then the addition of more stations near the boundary should be considered in order to resolve these features.

Because the coastline presents such a strong barrier to flow, most coastal flows are aligned along the coast. Thus stations oriented in a line perpendicular to the coast will generally sample the strongest hydrographic, and therefore biological gradients. Where the curvature of the coast is very sharp, a coastal current may separate from the coast and move into open water. The criterion for whether or not this separation will occur (Bormans and Garrett, 1989) is given roughly by the Rossby number, R_0:

$$R_o = \frac{u}{f r_c} .$$

Here u is the speed of the current, f is the Coriolis frequency for that latitude, and r_c is the radius of curvature of the coast (or the bathymetry). If this number can be calculated, and it is greater than 1, the coastal current is likely to separate from the coast and move offshore (Fig. 2.5).

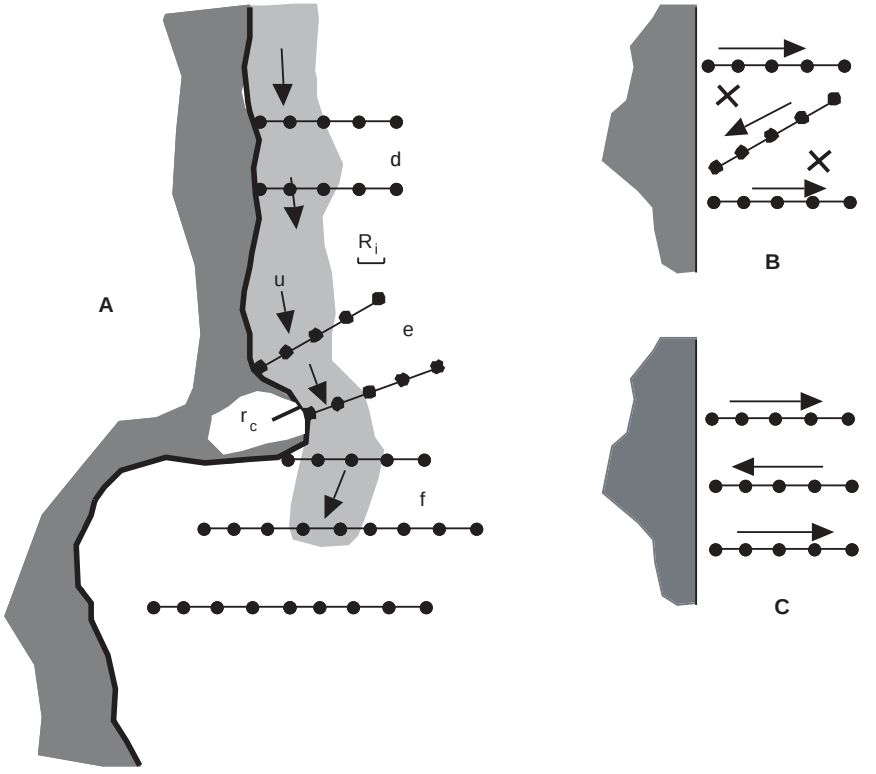

Figure 2.5
A, optimal station arrangement for sampling of a coastal current. In region (d), the coastline is straight, and transects should be parallel. In region (e), the coastline is bending, so transects should be oriented perpendicular to the shoreline. In region (f), the coastal current has separated from the coast as it passed the cape with radius of curvature, r_c. Transects here should be moved offshore in order to sample the coastal current in open water. B, transects arranged in a Z formation. The Xs indicate regions of low sample density. C, transects arranged in an E formation. Sample spacing is even.

If prior knowledge of the local harmful phytoplankton distribution is available, the sampling strategy may be tailored as follows. If harmful phytoplankton are known to be associated with the coastal current, the stations should be moved offshore in the lee of the topographic feature, in order to sample the waters that separated from the coast. On the other hand, if the harmful phytoplankton grow in the shelter of the topographic feature, for example in an eddy (Wolanski and Hamner, 1988), the stations should be moved inshore to follow the coastline.

If time and money permit, multiple transects should be run, ideally with the transects oriented parallel to each other. This will give even data coverage, with no poorly sampled areas (Figs. 2.5A, 2.5C). It may seem more efficient, in terms of navigation, to orient the transects into a Z shape. However, relatively large gaps in

coverage occur at the top and bottom of the Z (Fig. 2.5B). A more even coverage is obtained with an E-shaped cruise pattern (Fig. 2.5C).

Obviously, more transects will help to characterize a larger stretch of coastline, but as few as two transects may be sufficient to elucidate the location of source populations and transport processes from adjacent transects. For example, one might determine whether a coastal cell population originates in a river plume by locating one transect near the source of a river that forms a coastal current, while an adjacent transect is located out of the influence of the river source. Three or more transects will significantly improve the ability to resolve the larger alongshore patterns. The transect spacing need not be the same as the station spacing of the across-shelf transects, as changes alongshore are usually not as great as changes across the shelf. Without any prior knowledge of the cell distribution, a good rule of thumb is that the distance between adjacent transects should be some two to three times the across-shelf resolution. For example, a single transect may extend 40 km offshore, sampling every 5 km. Adjacent transects should then be spaced about 10–15 km from each other. These considerations are important when trying to contour the data and make surface maps of features. Numerical models can be useful tools to explore the adequacy of a sampling programme. In the Gulf of Maine, McGillicuddy (pers. com.) calculated an autocorrelation function from a model of dinoflagellate patches along the coast and showed that the across-shelf station spacing of 5 km was adequate to resolve patches, while the along-shelf spacing of 20 km was too coarse.

In the vertical, the most dense coverage possible is always preferable. Some workers advocate binning or 'pooling' of samples into three categories: below the thermocline, within the thermocline, and above the thermocline. While this approach serves to reduce the number of samples per station, we do not suggest this procedure for several reasons: (a) the thermocline may not correlate with the HAB species peak or the nutricline; (b) the sample resolution is low; (c) the variable depth of the thermocline will make data reduction and interpretation difficult; and (d) unexpected features may be missed. Rather, we recommend obtaining as many evenly spaced samples as is feasible given sample-processing time. The even spacing allows for quick and easy plotting of data, with good resolution of most vertical features. We suggest beginning with a vertical spacing no greater than 5 m, always collecting a surface or near-surface sample. Further refinement will be necessary depending on the distribution of the target species and hydrographic conditions. It is often possible to elucidate the relationship of harmful phytoplankton with water masses based solely on surface samples. However, subsurface populations may be extremely important in the dynamics, particularly in the early stages of a bloom, so caution should be exercised in interpreting surface data. Reduced vertical sampling should not be used until after a more thorough survey has allowed documentation of the subsurface patterns of phytoplankton and hydrography.

Often, the time necessary to sample intensively in the vertical will compromise the coverage in the horizontal. Broad horizontal coverage with somewhat limited vertical resolution may be the first step to identifying the general distribution of a toxic phytoplankton species. The timing and planning of these efforts should be co-ordinated with available data on toxicity patterns, and known associations of toxic phytoplankton with hydrographic features in the region. Once a population has been localized to a general area, much greater vertical resolution is needed to determine if there are significant populations that were not previously detected. Higher-resolution vertical sampling is required to study behavioural processes such as the

association with the nutricline or diel vertical migration behaviour of the population, or physical processes such as subduction of cell populations at fronts. It is often feasible to alternate cruise patterns to obtain dense vertical coverage along one transect during one cruise, and extensive horizontal coverage with coarse vertical resolution during another cruise. Alternatively, higher-resolution vertical coverage can be incorporated at a limited number of sites during a broad survey (i.e. one to two vertical profiles per transect).

The timing of sampling cruises will depend on many factors, including the organism being studied, the workforce available, the size of the boat, the oceanographic and meteorological conditions, and the amount of money available for cruises and data reduction. Two important generalizations can be made, however: (a) always begin sampling before a harmful species becomes dominant; and (b) sample as frequently as possible. The timing of cruises is determined by several factors including the growth rate of the phytoplankter, vertical migration behaviour, and the speed of alongshore currents. Cruises should be spaced so that a cruise is not totally independent of the previous one. For example, if a coastal current, u, is, on average, 10 cm s^{-1}, and R_i is 10 km, then cruises should ideally be $R_i/u \sim 1$ d apart if a single transect is sampled. If two parallel transects spanning 10 km are used, then cruises can be less often. This is seldom practical, so cruises are often one or two weeks apart, leading to problems in data interpretation. Again, the more extensive the spatial coverage of cruises, the longer the time interval can be between them. An alternate strategy is to undertake longer cruises which re-sample a grid pattern more than once. This gives important short-term information on the changes, which can supplement the data obtained over longer time intervals.

2.3.1 Ancillary data

2.3.1.1 *Shellfish toxicity data*

Any data available between cruises, such as shore-based sampling or mooring data, can be very useful in planning cruises or interpolating between cruises. In this regard, regional shellfish-monitoring programmes can be invaluable in providing a consistent temporal record of coastal toxicity. These programmes usually sample about once per week in the areas and during the times most prone to toxic events. The data can be quite useful in elucidating seasonal fluctuations that can guide cruise planning and reveal the timing of the blooms alongshore (Franks and Anderson, 1992*b*). Often the timing can be related to seasonal weather patterns where storms and downwelling favourable conditions may suggest the presence of offshore populations that are transported shoreward (Fraga *et al.*, 1988). Unfortunately, the monitoring programmes usually do not sample in the adjacent offshore waters. In one recent study, mussels (*Mytilus edulis*) were hung in bags on offshore hydrographic moorings and detected the transport of offshore *Alexandrium* populations toward the western Maine coast during downwelling favourable conditions (Anderson *et al.*, 2000). One advantage of using shellfish toxicity data is that the shellfish integrate the toxin over short timescales (one to two weeks) and can detect bloom events when shipboard sampling is not possible. However, one must be careful not to rely too heavily on shellfish toxicity data for several reasons: there may not be a sufficient number of cells present to cause toxicity or the indicator organism may not accumulate the toxin adequately. Alternatively, monitoring sites might not be ideally located to detect concentrations of toxic cells, particularly offshore blooms.

In the absence of a shellfish-monitoring programme, there are some environmental cues that correlate well with dinoflagellate blooms. As noted above, upwelling and downwelling favourable wind conditions can be associated with outbreaks of shellfish toxicity where surface blooms can be transported onshore during downwelling and offshore during upwelling. When sampling near a buoyant plume during downwelling conditions (see Fig. 2.4D), one sampling strategy might be to limit the extent of offshore sampling in favour of more alongshore transects as the plume is closer to the coast. The offshore extent should be increased during upwelling (see Fig. 2.4C). Alternatively, many dinoflagellate blooms are found near the pycnocline of a well-stratified water column. This stratification can be caused either by salinity differences or by heating. Thus strong rains, or several sunny days in a row can be important in bloom formation. Keeping a close eye on the weather is important when sampling for dinoflagellates.

2.3.1.2 *Satellite data*

The main utility of remote sensing is in providing a larger-scale synoptic picture than can be achieved through shipboard sampling. Satellite images are extremely useful tools in gaining understanding of the regional influences of processes affecting local phytoplankton populations. In particular, sea surface temperature (SST) imagery can aid in understanding the geographical extent of hydrographic features, the large-scale response of hydrography to forcings and the upstream conditions relative to a particular location. SST images give the boundaries of water masses; successive images may give some indication of water-mass movement, although currents along temperature gradients cannot be estimated.

The first study to use remote sensing to understand the distribution of toxic phytoplankton was probably that of Murphy *et al*. (1975). Several more recent studies of HABs have used remotely sensed images of sea surface temperature to aid in their prediction of transport of toxic phytoplankton (Johannessen *et al*., 1989; Tester *et al*., 1991; Franks and Anderson, 1992*a*; Keafer and Anderson, 1993; Stumpf *et al*., 1998). These images were useful only because the researchers had identified the association of noxious phytoplankton with particular water masses. One must be cautious in the interpretation of SST imagery as satellite sensors sample a very thin layer of water for temperature and so may not give an accurate picture of the absolute temperatures in a local area.

Unfortunately, toxic phytoplankton cannot be identified remotely; indeed, toxic outbreaks may occur with extremely low concentrations of cells ($<5 \times 10^2$ cells l^{-1}, e.g. *Alexandrium* blooms) which are difficult to identify even with a microscope. If a bloom is monospecific and occurs at relatively high abundances in the surface waters, then detection of species-specific chlorophyll *a* using ocean-colour imagery may be possible, but simultaneous ground truthing is required to ensure that the chorophyll *a* signal is related to the species of interest. Tester *et al.* (1998) estimated that about 10^5 cells l^{-1} were necessary for remote detection of *Gymnodinium breve* based on retrospective analysis of Coastal Zone Color Scanner data, a level that may give early warning of fish kills, but not shellfish toxicity. While the new ocean-colour sensors (e.g. SeaWiFS, launched in 1997; MODIS, launched in 1998; and MERIS, scheduled for 2001) promise greater spectral resolution and sensitivity, the ability to detect harmful species will be generally limited to situations where the surface signal from the HAB species is much greater than the ‘noise’ from co-occurring species and other non-biogenic materials in coastal waters.

Ocean colour can aid in the characterization of surface waters in much the same way that SST imagery has been used. For remote detection of a HAB species or the water masses in which they reside, one must be keenly aware of the physical and chemical environment as well as the physiology and behavioural characteristics of the species of interest (Tester and Stumpf, 1998). Knowledge of insolation, wind speed and direction, freshwater discharge and dynamic response to local topography are all essential elements in the interpretation of satellite images. Recent efforts have been made to integrate satellite imagery with these other data products and to provide those products over the World Wide Web and for geographical information systems (Eslinger *et al*., 1999). Remote-sensing data must be coupled with a basic understanding of the organism (or patterns of toxicity) and its habitat before being usefully implemented in a monitoring programme.

2.3.1.3 *Supplementary data*

Biological and hydrographic data gathered during a field programme indicate only the instantaneous state of the waters at the time of sampling. For information on the dynamic response, more consecutive data are necessary. In this regard, time series of wind speed and direction, and freshwater discharge of local rivers, may be invaluable. Wind speed and direction are often available from the local airport or national weather service (http://www.ncdc.noaa.gov/); while offshore buoy information can usually be obtained from national oceanographic data and buoy centres (http://seaboard.ndbc.noaa.gov/). Comparison of records from several locations along the coast should be made in order to assess the spatial and temporal scales of weather systems and correlation between locations. The stratification created by the low-salinity water can be an important habitat for dinoflagellates. Therefore, freshwater discharge rates from stream gauges are an important dataset along any coastline that has a reasonably sized river and are usually available from government water resources authorities (http://water.usgs.gov/). Continuous records of tidal height and bottom pressure are often made along coastlines. This information can be used to infer geostrophic currents or propagation speeds of water masses along a coast (Brown and Irish, 1993) and is occasionally available at oceanographic data centres (http://www.nodc.noaa.gov/).

2.4 Data interpretation

Once biological and hydrographic data have been gathered, it is necessary to process and collate them in a form that will make dynamics and correlations apparent. Three plotting formats are central to this data reduction: vertical profiles (Fig. 2.6), temperature *v.* salinity (TS) plots (Fig. 2.7), and contour plots (Fig. 2.8). Profiles are plots of properties determined by vertical sampling at a given station (Fig. 2.6). Associations between variables at a station can be assessed by plotting several variables on the same set of vertical axes. Thus subsurface peaks in species concentrations may be seen to be associated with the shallow diurnal pycnocline, for example. Or a lack of association of harmful species with the vertical profile of chlorophyll may become apparent.

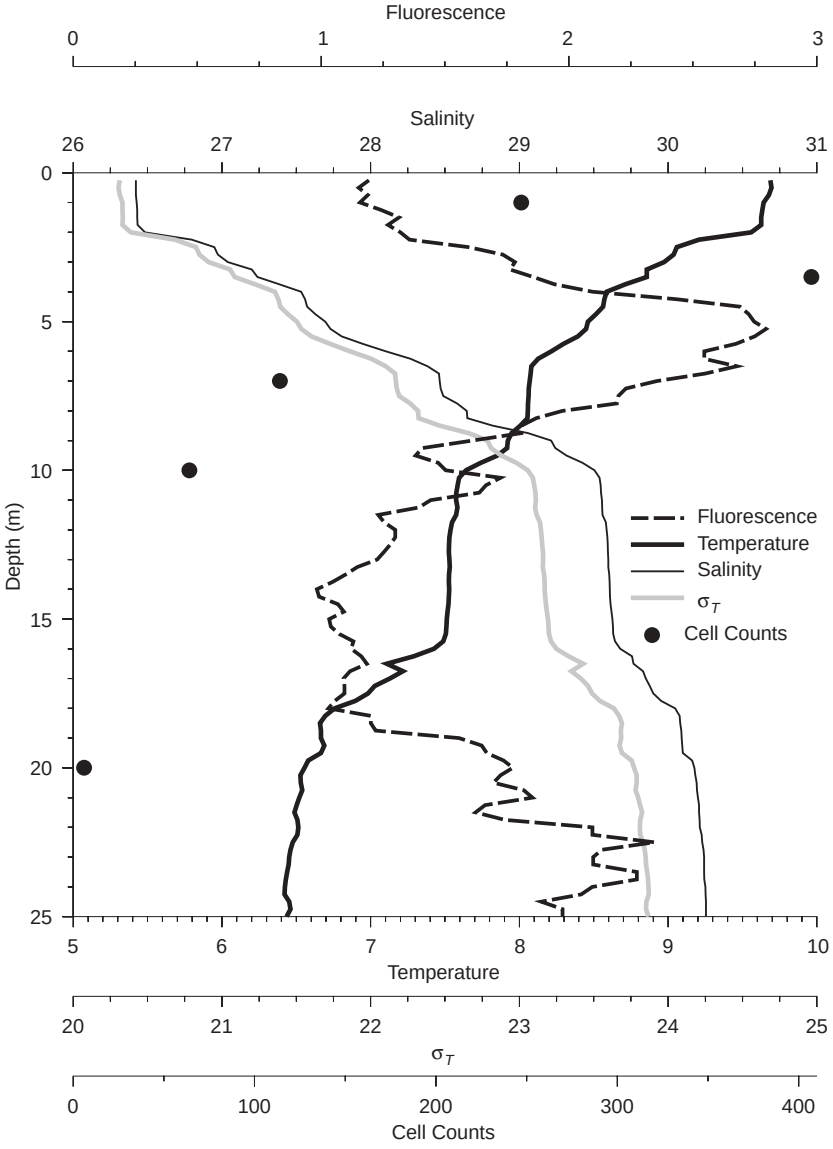

Figure 2.6
Example of a vertical profile plot, with five variables (cell density, salinity, temperature, density, fluorescence) all plotted on the same vertical axis.

On a TS plot, lines of constant density appear as curves generally stretching from lower-left to upper right for most ranges of T and S. Water masses have characteristic TS values; mixing between two water masses gives waters with properties that form a straight line joining the two source water masses. By following TS values

that form straight lines on the TS plot, inferences about source waters and mixing can be made. Seven water masses have been labelled in Fig. 2.7 (I–VII). Waters along the trajectory I–IV–V are mixtures of warm freshwater (I) with colder, saltier water (V). Waters along the lines II–IV and III–V are formed from waters more saline than I mixing with waters formed by mixtures of I and V. The waters along V–VI and VI–VII are deeper, colder waters that are probably present all year round. The warmer, fresher waters (I, II, III and IV) are probably seasonal waters formed from freshwater runoff and solar heating. Note that heating of waters (without mixing) would cause the curves to extend vertically with no change in salinity. The fact that there are very few points that do this suggests that mixing of surface (I, II and III) and deeper (IV, V) waters has occurred during or after solar heating.

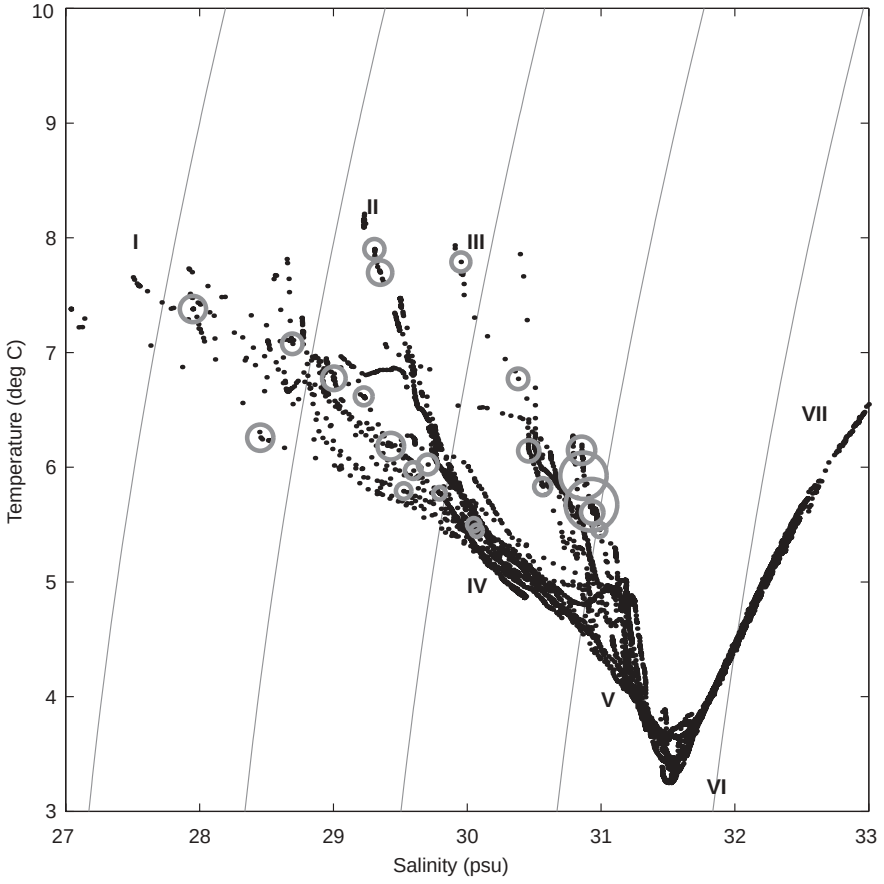

Figure 2.7
Temperature v. salinity plot. Data showing the temperature and salinity properties of the water (black dots) with the presence of toxic *Alexandrium* cells (grey circles – diameter proportional to cell concentration). Grey lines are contours of density. Water masses are indicated with Roman numerals
I–VII. Mixing of water masses (e.g. I–IV, VI–VII, etc.) occurs along straight lines in a TS plot.

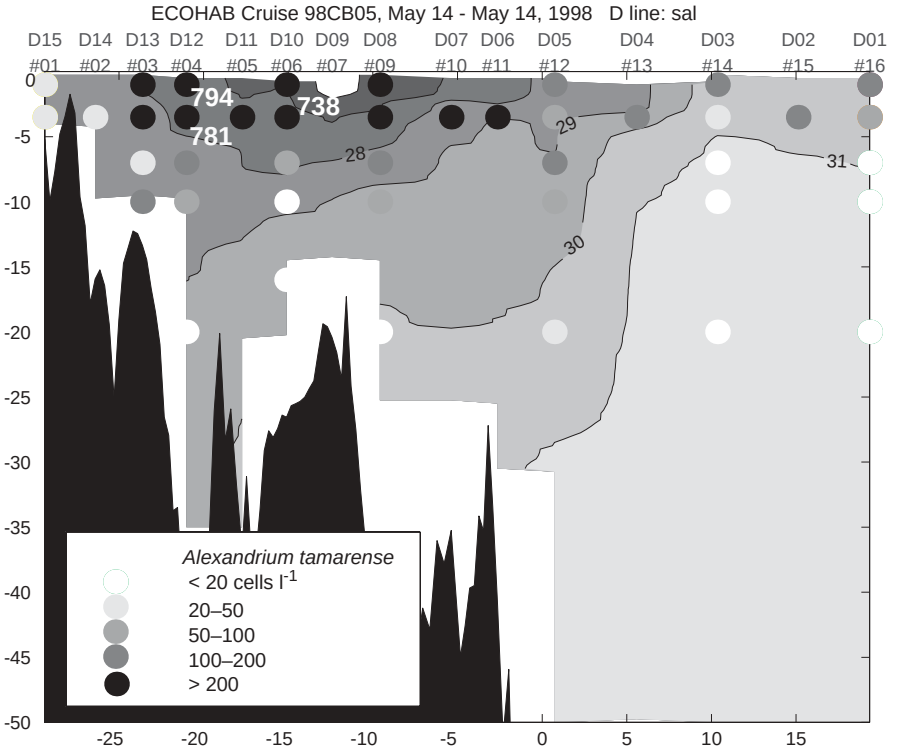

Figure 2.8
Data from 15 stations (D01–D15) extending from the coast of Maine (USA) into the Gulf of Maine, contoured into a cross-sectional view. Two properties have been plotted on the same axes: salinity and cell counts. The sloping isohalines (solid black lines) indicate that there is a buoyant plume propagating near the coast. The slope of the isohalines suggests (via geostrophy in the Northern Hemisphere) that the water currents are off the graph at stations D03–D10. The highest cell densities (shades of grey) are associated with the lower salinity water, indicating that the cells are being advected alongshore (off the graph) with the water. Source : Anderson and Keafer, unpublished data.

As the points on Fig. 2.7 were derived from widely spaced vertical profiles, not much information is available on horizontal mixing in these waters. It is certainly possible that waters I, II and III form a continuum of nearshore freshwater (I) mixing with offshore salty water (III) at about 8°C. The near-surface waters have mixed vertically with deeper waters, forming the lines I–IV–V, II–IV and III–V. Plotted along with the TS values are *Alexandrium tamarense* cell concentrations. The distribution of high cell counts is difficult to interpret because of cell growth since mixing, uncoupled from the mixing itself. Thus the highest cell concentrations may not correspond to source waters if the cells grow faster in new waters formed through mixing. Clearly the cell concentrations are highest near the surface, and there is

some indication that cells had a low-salinity source (note the high concentrations along I–IV). The high cell concentrations along III–V – offshore surface waters mixing with deeper waters – are more difficult to explain. They could originate from cells inoculated into these waters when they were formed (a local source with *in situ* growth), or they could have come from lateral mixing of nearshore freshwaters followed by growth. The lack of horizontal resolution of the samples confounds the interpretation.

Contour plots address the limitation of horizontal resolution of TS plots by providing a two-dimensional view, in either a vertical or horizontal plane, of the variability of properties along two spatial axes (Fig. 2.8). Whereas profile plots may show an association of toxic cells with the pycnocline, contour plots in a vertical plane may show the pycnocline to have a slope, indicating a flow perpendicular to the plane. From this we could infer that the toxic cells are being transported in that direction. Contour plots can be even more useful than profile plots in showing the relationship between two variables. By plotting contours of the two variables on the same axes, and overlaying the plots, a two-dimensional picture of the patchiness and spatial correlations between properties becomes apparent. Many computer programmes are available for making contour plots, but these packages have their limitations and are notorious for drawing 'bull's-eyes' around high data points. Higher spatial resolution of the sampling programme minimizes these problems. Drawing contours by hand at least once is a good exercise for any scientist so that one can be confident that the contouring software is deriving the most realistic pattern.

2.5 Discussion

Several studies of harmful algal blooms have brought together many of the sampling techniques and strategies described above to elucidate the factors leading to local outbreaks and transport mechanisms in coastal regions. Good examples are the studies by Tyler and Seliger (Tyler, 1984; Tyler and Seliger, 1978; 1981) of *Prorocentrum mariae-lebouriae* in the Chesapeake Bay estuary (USA), the field work by Franks and Anderson (Franks and Anderson, 1992*a*, 1992*b*) examining *Alexandrium tamarense* in the Gulf of Maine (USA), the work by many scientists to determine the factors leading to the 1988 *Chrysochromulina polylepis* bloom along the Scandinavian coasts (Dundas *et al*., 1989), and the studies of *Gymnodinium catenatum* and *Alexandrium affine* in the rias of Spain (Fraga *et al*., 1988). While this list is certainly not exhaustive, it is representative of the types of study that can ultimately lead to some limited predictive capability for toxic phytoplankton blooms.

A feature common to all these studies is that they incorporate a variety of databases and types of information. While a single cruise may not yield much information, several cruises under different conditions, along with good insights into the local physical forcings, supplementary data to interpolate conditions between cruises, and a variety of biological samples, allow the researchers to formulate strong, testable hypotheses from which the course of future blooms may be predicted. A finding common to most, if not all studies of harmful algal blooms is that their occurrence is strongly linked to recent and prevailing meteorological conditions. This immediately suggests that blooms are probably no more predictable than the weather, limiting their forecast to, at most, about a week. This inherent limitation in the predictability of harmful algal blooms demands that sampling be done on a

short enough timescale that important events are not missed. This strong constraint will largely determine the timing of cruises, and arrangement of sampling stations.

While the sampling strategies described here may aid in the determination of how a toxic bloom arrived at a given locale along a coast, they will not necessarily allow identification of the processes that began the bloom. Steidinger (1983) suggests four stages for a phytoplankton bloom: initiation, growth, concentration, dispersal. The sampling techniques described here will aid in elucidating the factors leading to growth, concentration and dispersal. The factors allowing the initiation of a harmful algal bloom fall squarely within the realm of the physiologist, who can give information concerning growth response to temperature, salinity, light, nutrients, turbulence, and life-history traits such as excystment or sexual stages. However, it is the transport of harmful blooms along coastlines that can lead to widespread ecological devastation, economic hardship and health risk. It is imperative, therefore, that we design and implement useful and efficient coastal-sampling schemes, in order to relate local harmful phytoplankton blooms to the physical systems that influence their distribution.

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3

Culture methods

R R. L. Guillard and S. L. Morton

This chapter deals with the culture of marine microalgae (phytoplankters) of known or presumed toxicity or harm to man or to animals of commercial interest. Most phytoplankters suspected of producing bioactive substances are oxygen-evolving phototrophs, thus this chapter deals largely with autotrophic and auxotrophic modes of nutrition. However, in recent years a number of heterotrophic and mixotrophic species, primarily dinoflagellates, have been found to produce bioactive compounds and to be linked to fish mortalities and human illness.

For some research it is sufficient to use strains of microalgae already in culture. Some of these may be obtained from various sources (universities, research institutes, government laboratories, formal culture collections) (see Appendix A). It is usually desirable and often essential to obtain cultures of harmful or nuisance species from local waters they infest. Such cultures not only help in the identification of the organisms, but also can be critical in determining the systematic position of a previously unrecognized organism. Pigment content, ultrastructure, genetics, life cycles, storage products, nucleic acid contents, toxin production, and toxin identification are among the properties that can be studied in culture. Any attempt to correlate the occurrence of a species with the ecology of its habitat through culture experiments should obviously employ strains from the local population of interest.

The process of establishing unialgal cultures from natural populations has special requirements; cell numbers are usually small and nutrient levels sometimes are low. The composition of the nutrient mixture used either for enrichment of natural water samples containing the organisms of interest or for direct isolation of single cells or colonies must favour survival and continued cell division. With rare exceptions, the nutrient additions for isolations are dilute compared to those of ‘grow-out media’, and varied in nature. In isolation work the final stoichiometry of cell contents is not the issue. Nutrient ratios, when important, are so because they influence absorption by the cell or affect ionic speciation in the medium; the later particularly in the case of trace metals and chelators. On the other hand, for growing crops of phytoplankton, natural seawater contains an abundance of many elements, but far too little of others. Thus, various enrichments of seawater have been designed to supply the scarce material in usable forms at levels that are neither toxic nor growth-rate limiting.

3.1 General laboratory procedures

Phytoplankters isolated into culture will proceed though the typical growth pattern of lag phase, log phase, stationary phase and senescence phase. A population started

from a single specimen isolated from nature is allowed to increase in a favourable environment until some factor, usually nutrients or light with autotrophic organisms or prey availability with heterotrophic organisms, stops cell multiplication. Once a culture reaches mid-log phase growth, a daughter culture is inoculated from the parent culture under aseptic conditions. The parent culture is then kept as a 'back-up' and as a source of inoculum for parallel cultures intended for experiments or crop production. The critical point is to keep one continuous line of culture growing vigorously, uncontaminated, and transferred before its 'half life' is past. The parent and daughter cultures should be stored in separate growth chambers in case of chamber failure. Some species can be stored as cysts, spores, resting cells, or even in cryogenic storage, but this is not the usual practice with strains in active use. In order to avoid contaminating local waters, all cultures of known or presumed toxic species should be kept and discarded with precautions like those used for pathogens. Discarded cultures and supernatants of cell harvests should be autoclaved or chemically sterilized with sodium hypochlorite before being released into waste lines.

3.1.1 Bench facilities

Most manipulations can be carried out on any desk height bench top that has space for the knees beneath it. Ideally, a laminar flow hood that produces a sterile air environment should be used for transferring hazardous cultures. The filters of a laminar flow hood should be checked periodically for sterility, and certified. Alternatively, a simple hood shown in Guillard (1995, Fig. 3.1) can help to reduce contamination from the air.

3.1.2 Culture vessels, enrichment

For enrichment or selective cultures made from water samples, the liquid volumes should not be less than 100 ml for small species and should be at least 1 l for large species. Glass or polycarbonate Erlenmeyer flasks of 250 ml to 2 l are suitable, covered by cotton plugs or inverted beakers of glass or polypropylene. Glass or polycarbonate bottles or Fernbach flasks (2,800 ml) are also satisfactory for large volumes. The liquid/air surface should be large. Cleaning culture vessels involves washing in detergent, followed by scrubbing with a brush when necessary to dislodge solid algal remains or other material, then rinsing with tap water, dilute HCl (10%, which is 1.2 N) to remove carbonate deposits, then a final rinse with tap water and 'purified water' (distilled or deionized). The final rinsing with tap water referred to above should include filling and emptying smaller vessels four to six times and swirling larger vessels with several changes of smaller volume.

3.1.3 Isolation tubes, culture tubes, culture flasks

Borosilicate tubes, screw-capped or with slip caps, are usually used for both single-cell isolation work and dilution cultures. Some collections maintain their cultures in tubes to conserve space. Those are usually 16×120 mm or 20×150 mm in size. Caps should be Teflon-lined or made from polypropylene. The 25×150 mm (or 20×150) threaded tubes are adequate for small-volume maintenance cultures of certain small flagellate species. Flat-bottomed tubes are best. The standard maintenance vessels have for years been cotton-plugged 125 ml borosilicate flasks containing 40–50 ml of autoclaved medium. Good surface-to-volume ratios are provided by

100 ml of liquid in 250 ml flasks and 1 l in 2 l flasks. Polycarbonate Erlenmeyer flasks have excellent properties but cannot be autoclaved often with seawater in them because of the high pH reached during autoclaving.

3.1.4 Pipettes

For transferring stock cultures or starting cultures used as inoculum, sterile cotton plugged 230 cm (9 inch) Pasteur pipettes or disposable pre-sterilized pipettes (1 ml or 2 ml) are satisfactory. For larger quantitative inoculation of experiments or for starting cultures of larger volumes, calibrated pipettes (up to 100 ml) can be used.

Isolation pipettes for cells or colonies are made by drawing out sterilized 230 cm Pasteur pipettes over a flame into capillaries of diameter suited to the species involved (Hoshaw and Rosowski, 1973, Fig. 3.3). Fig. 3.1 shows the general construction of an isolating pipette with mouthpiece tube. If for some reason the mouthpiece tube cannot be used, employ a heavy walled bulb made from tubing on the drawn-out pipette (see Droop, 1954, for method). Some workers use drawn-out capillary tubes for isolations, attached to thin tubing.

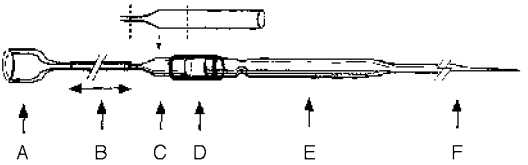

Figure 3.1
Diagram of isolating pipette mouthpiece tube: A, mouthpiece as used for blood diluting pipettes, or improvised; B, latex tubing 1/8 inch (3.2 mm bore, 1/32) (0.8 mm wall thickness, 30–40 cm long); C, portion of Pasteur pipette, scored with a file and broken off; D, latex tubing 1/4 inch (6.4 mm inside diameter), 1/16 inch (1.6 mm wall thickness, 12–15 mm long); E, drawn-out Pasteur pipette being used for isolation; F, capillary end of pipette.

3.1.5 Other material for pipette isolations

Small cells can be isolated from and washed through drops of water using 12-well borosilicate glass commercial chemical spot plates, or on depression slides (25×75 mm, two or three depressions per slide), on ordinary glass slides, or in opened disposable plastic Petri dishes. A bunsen burner or other flame source, fine and medium forceps, wire tube racks, and tape for labelling tubes and flasks are needed. For further description of useful materials and arrangement of facilities see Hoshaw and Rosowski (1973) or Guillard (1973, 1975, 1983). Plankton nets and sieves made from nylon plankton netting (see Tangen, 1978, for sources) glued to sections of plastic pipe are widely used, either to get rid of the larger species in order to deal with the smaller ones, to wash away the smaller ones, or just to remove the larger grazers. Reverse (upward) filtration exerts very little pressure on the cells being concentrated (Dodson and Thomas, 1964). Polycarbonate filters with holes of 0.8–12 µm can serve the same purpose as nets for separating small cells. Small contaminating cells such as those of *Synechococcus or Nannochloropsis* can sometimes

be greatly reduced in numbers relative to the species desired by washing them through the filters using repeated additions of sterile medium (the filter surface must not be allowed to dry). Centrifugation, repeated when tolerated, and using sterile seawater or medium, can help to separate cell types and can reduce bacterial numbers preliminary to purification efforts. Methods for isolating dinoflagellate cysts are discussed by Anderson *et al*. (this volume).

3.1.6 Optical equipment

Any compound microscope, preferably with phase contrast, is suitable for general observation. Isolation is almost always done using a stereomicroscope that has a base for transmitted illumination and a mirror that can be rotated. It is possible to isolate cells only 2–4 µm in size with a stereomicroscope using darkfield illumination. The magnification used for isolation seldom exceeds 80x, but good resolution is desirable for distinguishing cells from detritus.

3.1.7 Filtration

Filtration has three different purposes in algal culture. The first, generally using glass-fibre filters, is to remove detritus and larger planktonic organisms from the seawater. The second purpose is to provide diluent either for serial dilution isolations or for dilution of seawater samples brought into the laboratory for acclimation or selective enrichment prior to isolation efforts. For this purpose filtration may be through membrane filters of 45–50 mm diameter and about 0.4 µm porosity, which permit only some heterotrophic bacteria and prochlorophytes to pass. Membrane filters of 0.22 µm porosity are generally considered to yield water free of bacteria, but not of viruses. The third purpose for filtering is to sterilize liquids that should not be subjected to autoclaving. A suggested convenient large-volume set-up is shown in Guillard (1995, Fig. 3.3).

3.1.8 Apparatus for sterilization by heat

A commercial autoclave is best, but pressure cookers of various sizes are also suitable. Sterility requires 15 minutes at 121°C in the entire volume of liquid. Maximum-indicating thermometers are available. Routine culture safety is helped by using indicator tapes that change colour upon autoclaving to identify flasks, pipette tubes, etc. that have been sterilized. Heating to 90–95°C for at least 30 min, and cooling, and repeating on two successive days is an alternative process ('tyndallization'). It is assumed that vegetative cells are killed by heat and that heat-resistant spores will germinate in the following cool periods and be killed by subsequent heatings.

Keller *et al*. (1988) and Price *et al*. (1989) have explored sterilization by commercial microwave apparatus. The method is well suited to treating 2 l Teflon or polycarbonate bottles (up to three at one time in the microwave) holding 1.5 l of seawater or medium. Check the protocol used (timing, etc.) for sterility efficiency. Some materials cannot be microwaved without damage.

Autoclaving medium is still the ultimate guarantee of sterility; it includes the destruction of viruses. Autoclaving in Teflon would reduce losses and avoid leaching of materials (silicon especially) from glass. However, care must be taken to ensure that the temperature within these bottles reaches 121°C for the appropriate amount

of time due to the slow rate of heat transfer of Teflon. In many media, including L1 (see below), a precipitate forms upon autoclaving, some of which may disappear upon re-equilibration of the liquid with CO_2 in the air. To minimize precipitation, remove medium from the autoclave as soon as pressure is down and temperature in the autoclave has fallen to about 80°C ; avoid moving superheated medium because bubbling causes loss of CO_2 and thus raises the pH of the water.

3.1.9 Illumination

The problem of temperature control in a culture facility may influence the lighting technology chosen. Important considerations regarding light are intensity (irradiance), colour (spectral distribution) and duration (day length). Daylight does very well for most cultures, but the intensity, spectrum and day length are dependent on geography and weather. Screens or gauze will attenuate daylight if needed. Maximum irradiance experienced by cultures during the day should be 85–170 µEinstein $sec^{-1}m^{-2}$. Many algae grow well at 10–30 µEinstein $sec^{-1}m^{-2}$ (1–2% full sunlight), and some only then; back-up cultures always survive better at low intensities. It is noteworthy that a Pacific strain of the pelagic cyanobacterium *Trichodesmium* initially grew successfully under dim daylight (10 µEinstein sec^{-1} m^{-2}) and could not be grown under fluorescent light (Ohki and Fujita, 1982; Ohki *et al.,* 1986). However, once in culture, this strain could be grown at 20 µEinstein sec^{-1} m^{-2} provided by fluorescent bulbs (Mulholland *et al*., 1999). Natural light may have too much red and far-red light for algae that live in deep water (unfavourable red to blue ratio: Brand, 1986). Fluorescent bulbs remain the light source of choice, employing coloured filters when necessary. The 'cool white' type is somewhat favoured because of a better ratio of light output to energy consumed and general availability. Its spectral distribution of energy is reasonably good for coastal species and perhaps others living in surface waters. 'Daylight' bulbs have more of their output in the blue, but less total light. Fluorescent bulbs emit roughly 2% of their radiant energy in the near-ultraviolet (<380 nm), and evidence suggests that this can inhibit dinoflagellates (reviewed by Guillard and Keller, 1984). If this proves to be a problem, polycarbonate or lucite can be used to absorb ultraviolet. While it is true that some algae tolerate or thrive in continuous light, many species, especially dinoflagellates, have an absolute requirement for a dark period, even if the irradiance is low (Brand and Guillard, 1981). Light/dark cycles of 16 h/8 h, 14 h/10 h, and 12 h/12 h are commonly used. Day length is particularly important for the necessary sexual reproduction in culture of pennate diatoms, notably the toxic *Pseudo-nitzschia* (Hiltz *et al*., 2000).

3.1.10 Temperature control

Uncontrolled room temperature is seldom satisfactory for marine algae. Rooms and culture boxes should have safety systems to guard against overheating or chilling (recommendations in Guillard, 1975 or 1983). Excursions of temperature, sometimes too low, but usually too high, cause by far the most losses of cultures. It is most important to have alternative safe locations.

3.2 Isolation procedures – sampling and incubation

Fig. 3.2 outlines possible treatments of collected samples (3.2A). Water samples should be collected in clean vessels (*c.* 1 l) and protected from bright light and changes of temperature. The well-known agar plate techniques of streak plating and spray plating are well described by Hoshaw and Rosowski (1973) and can be applied to such nuisance genera as *Nannochloropsis, Nannochloris, Chlorella* and possibly *Pseudo-nitzschia*. Applying screening before steps 3.2D or E. to remove larger predators is always desirable because populations of desired cells can be depleted in just hours. Predator removal is essential in paths 3.2B and C. Selective screening is often helpful via any path to reduce the numbers of organisms larger or smaller than the ones desired. At any of the steps 3.2B, C, D or E, the whole algal population, some part of it, or single cells, will have been put into a limited volume of culture and moved into a new physical environment. Light and temperature are the manipulable physical factors that can change the rates or timing of cell processes. The ideal for protocols B and especially D and E would be to duplicate natural circumstances unless there were a priori knowledge that something was less than favourable at the time of collection. Salinity is not usually changed from that of the sample. Guillard (1973) describes the basic dilution culture technique for isolation while Andersen and Throndsen (Chapter 4) describe the basics of plankton enumeration by this method. For heterotrophs it may be necessary or desirable to add cultured cells of species known or suspected to serve as food for the predator desired. The food source is applied before steps 3.2B, C, D or E.

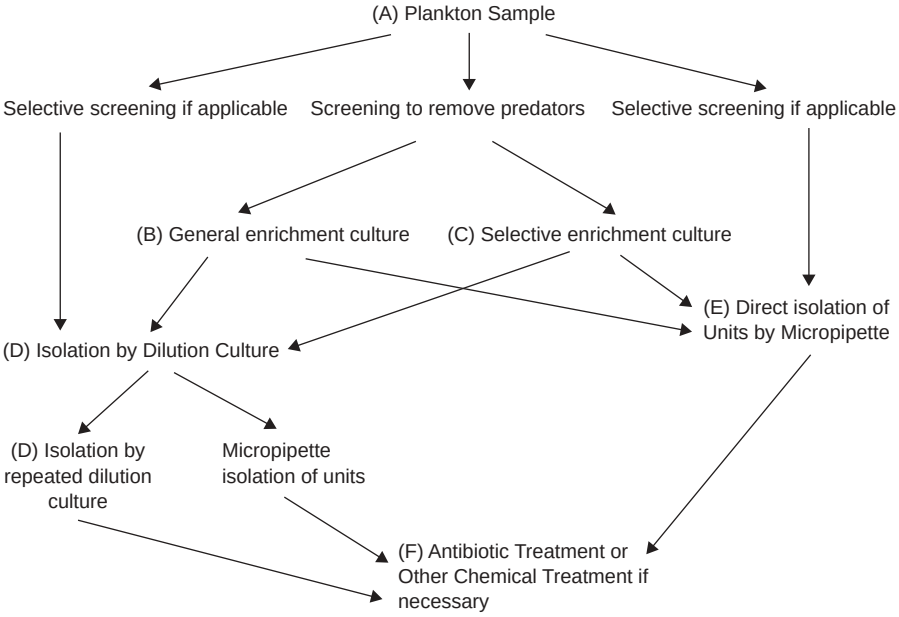

Figure 3.2
Alternative treatments of a phytoplankton sample collected for establishing cultures derived from single cells or colonies.

3.3 Manipulations – enrichment cultures

3.3.1 General enrichment cultures

General enrichment cultures (Fig. 3.2B) are intended to support the entire phytoplankton population to provide adequate material for isolation by protocols D or E. The following nutrient additions are recommended, concentrations not to exceed those given: (inorganic) nitrate, 35 µM; ammonium (NH_4Cl), 10 µM; urea, 10 µM; sodium glycerophosphate, 3 µM; silicate (for diatoms or silicoflagellates only) 50 µM; vitamins, 1/10 the level in L1 (see Tables 3.1 and 3.2). For coastal and oceanic upwelling waters use the L1 chelated trace-metal mix at 1/10 level. For oligotrophic ocean waters use the L1 mix at 1/100 the L1 level but add additional EDTA (ethylenedinitrilotetraacetic acid) to 10^{-6} M. Alternatively the L2 trace-metal mix (Table 3.3) at the 1/100 level can be used. Because inorganic phosphate salts have occasionally been found to be inhibitory, presumably because of impurities, glycerophosphate is suggested (see Dzurica *et al*., 1989, for *Aureococcus anophagefferens* and Ohki *et al*., 1986, for Pacific isolates of *Trichodesmium* species). However, purified grades of phosphate salts are usually tolerated at micromolar levels. Soil extracts are called for in some recipes and often found of benefit in isolation efforts. One procedure for making soil extract is essentially that of Gross (1932). For soil, use a good garden soil not recently fertilized, or a commercial potting soil, which may be easier to obtain in some locations. Autoclave (15–30 min) approximately equal weights of soil and (fresh) water and allow to stand a few days for sediment to settle to some extent. Clarify by decanting, filtering or centrifuging (or all three). The extract (supernatant) is re-autoclaved if necessary and stored sterile in the cold. Use 10–50 (usually 20) ml l^{-1} of final culture medium.

3.3.2 Selective enrichment cultures

Selective enrichment cultures (Fig. 3.2C) employ either physical or nutritional treatments to influence differentially the growth rates of various species in the sample. Hundreds of combinations are thus possible but logistically unmanageable, calling for educated guesses based on observations of the natural population. Nutritional aspects are easier to predict in some cases. Diatoms can be suppressed by omitting silicate and incubating in polycarbonate; if the sample water is very high in silicon then 1–10 mg l^{-1} (10^{-5}–10^{-4} M) of germanium dioxide may be used to inhibit diatoms selectively (also silicoflagellates, presumably). Omitting ammonium will discriminate against species that cannot use nitrate, such as certain cryptomonads, euglenoids and chlamydomonads. Some of these species tolerate ammonium concentrations toxic to most other algae – as high as 0.5 µM. However, many species that absolutely require ammonium (or other sources of reduced nitrogen) are poisoned by levels as low as 25–50 µM. Thus ammonium (NH_4Cl) addition can be a positive selective agent at low levels (*c.* 25 µM) or a negative one, even at levels below 100 µM. Omitting nitrate, in the presence of ammonium, will help to select for species that require ammonium. Additions of glutamate, arginine, asparagine or other organic nitrogen compounds (10^{-6} to 10^{-8} M) are potentially useful (Mihnea, 1993; Dzurica *et al*., 1989). Strains of *Synechococcus* may develop as contaminants in cultures of eukaryotic species when rigorously ‘clean’ techniques are used; low levels of streptomycin (<25 mg l^{-1}) or penicillin eliminate them (as well as other cyanobacteria). Other selective treatments are referred to in Guillard (1973).

TABLE 3.1 Composition of L1, an enrichment of seawater[a]

Major nutrients			
Compound	Working stock[b] (g l^{-1})	Final concentration (mol l^{-1})	Final concentration in seawater medium[c] (mol l^{-1})
$NaNO_3$	75	8.83×10^{-1}	8.83×10^{-4}
NaH_2PO_4 H_2O	5	3.63×10^{-2}	3.63×10^{-5}
Na_2SiO_3 $9H_2O$[d]	30	1.07×10^{-1}	1.07×10^{-4}
Trace elements			
	Working stock (g 100 ml^{-1})	Primary stock (mol l^{-1})	Working stock[b]	Final concentration in seawater medium (mol l^{-1}) (rounded)
$FeCl_3$ $6H_2O$[e]	–	–	3.15 g l^{-1}	1.17×10^{-5}
Na_2 EDTA $2H_2O$	–	–	4.36 g l^{-1}	1.17×10^{-5}
$MnCl_2$ $4H_2O$	18	9×10^{-1}	9.0×10^{-4} M	9.0×10^{-7}
$ZnSO_4$ $7H_2O$	2.20	8×10^{-2}	8.0×10^{-5} M	8.0×10^{-8}
$CoCl_2$ $6H_2O$	1	4×10^{-2}	4.0×10^{-5}	4.0×10^{-8}
$CuSO_4$ $5H_2O$[f]	0.25	4×10^{-2}	1.0×10^{-5} M	1.0×10^{-8}
Na_2MoO_4 $2H_2O$[f]	1.89	9×10^{-2}	9.0×10^{-5} M	9.0×10^{-8}
H_2SeO_3[g]	0.13	1×10^{-2}	1.0×10^{-5} M	1.0×10^{-8}
$NiSo_4$ $6H_2O$	0.27	1×10^{-2}	1.0×10^{-5} M	1.0×10^{-8}
NH_4VO_3	0.12	1×10^{-2}	1.0×10^{-5} M	1.0×10^{-8}
K_2CrO_4	0.0194	1×10^{-3}	1.0×10^{-6} M	1.0×10^{-9}
Vitamins[h]				
Thiamine HCl			100 mg l^{-1}	3.0×10^{-7}
Biotin			500 µg l^{-1}	2.1×10^{-9}
B_{12}			500 µg l^{-1}	3.7×10^{-10}

a. This version of the f enrichment has additional trace elements and changed levels of Cu and Mo. Complete directions for preparing f/2 and comments and other modifications are given in Guillard (1975, 1983).
b. Make stock in distilled or deionized water.
c. Natural seawater of appropriate salinity or suitable artificial seawater base. Use 1 ml of all working stocks per litre of final enriched seawater.
d. Add silicate only for diatoms or silicoflagellates.
e. Separate iron and EDTA additions can be replaced by 5 g l^{-1} of ferric EDTA (13% Fe) as described in the f/2 formulation.
f. Working stock of trace elements is made by adding EDTA and iron (or ferric EDTA) first, then using 1 ml l^{-1} of all the L1 trace-element primary stock solutions. If f/2 primary stock solutions are available, use the f/2 Cu primary stock at 0.25 ml l^{-1} of L1 working stock and the f/2 Mo primary stock at 3 ml l^{-1} of L1 working stock. The Fe, EDTA, Mn, Zn and Co levels in f/2 and L1 are the same.
g. Equivalent concentrations of anhydrous sodium selenite (formula weight 172.94) or sodium selenite pentahydrate (FW 263.02) may be used in the primary stocks instead of selenious acid.
h. Directions for making vitamin stocks are given in Table 3.2.

Source: Guillard and Hargraves (1993).

TABLE 3.2 Vitamin primary stock solutions and working stock solutions[a]

Primary stock solutions: Biotin is obtained in crystalline form; allow for about 4% water of crystallization. A primary stock solution is made containing 0.1 mg ml^{-1} by weighing about 10 mg and adding distilled water, 9.6 ml for each milligram of biotin. Make the solution slightly acid if it is to be autoclaved. Keep it sterile and frozen.

Vitamin B_{12} is similarly obtained as crystals, and 11% should be allowed for water of crystallization. Weigh and make a primary stock solution having 1 mg ml^{-1}. Acidify the solution if it is to be autoclaved, and keep it sterile and frozen.

Primary stocks can be put up in ampoules, screw-capped test tubes or flasks.

Working stock solution: Bring 1.0 ml of biotin primary stock and 0.1 ml of B_{12} primary stock to 200 ml and add 20 mg of thiamine HCl. No primary stock of thiamine HCl is needed.

The vitamin stock solution is dispensed in 1.2 ml or 5 ml lots in ampoules or in 10 ml lots in screw-capped test tubes, autoclaved, and then stored in a refrigerator. Use 1 ml l^{-1} of final medium. Vitamins are usually added before autoclaving. Keep open containers sterile or refrigerated.

a. Made according to the f/2 directions (Guillard, 1983) except at half that concentration so that all L1 working stocks are used at 1 ml l^{-1}. (The f/2 vitamin stock was used at 0.5 ml l^{-1}.)

TABLE 3.3 Enrichment L2

Algal enrichment L1[a]	1 l
Na_2 EDTA $2H_2O$[b]	32.86 mg

a. seawater algal medium L1 according to Table 3.1.
b. Prepare a stock solution of 3.286 g Na_2 EDTA $2H_2O$ per 100 ml of distilled or deionized water and use 1 ml per litre of final L2 to yield 10^{-4} M EDTA. Alternatively, a separate L2 metal stock solution can be made with the metals as shown in Table 3.1 but with Na_2 EDTA increased to 37.22 g per litre in the working stock.

3.3.3 Special factors in enrichment

Trace elements, chelators and vitamins call for special attention when making enrichment cultures. Selenium, molybdenum and vanadium are influenced little if at all by chelation. Selenium, as selenite (SeO_3^{-2}) is present in (undiluted) L1 medium at 10^{-8} M, almost ten times the content of all selenium species in average seawater, estimated at 1.7×10^{-9} M (Brand, 1986). There is reason to suspect that for some species a 10-fold dilution can result in growth rate or crop limitations, or both. Species of the prymnesiophyte genus *Chrysochromulina* have long been known to have high Se requirements (see Granéli *et al*., 1993; Harrison *et al*., 1988). The 'brown-tide' organism *Aureococcus anophagefferens* in citrate-chelated artificial seawater 'Aquil' showed crop limitation below 10^{-8} M Se (at low Fe) and 10^{-9} M Se (at 10^{-5} M Fe). Growth rate was markedly enhanced at high Se plus high Fe (Cosper *et al*., 1993). As a general rule it is prudent to keep added selenite at around 10^{-8} M to 10^{-9} M even in enrichment and dilution cultures.

Undiluted L1 medium contains molybdenum at about the average molar concentration in seawater – estimated as 1.1×10^{-7} M (Brand, 1986). Cobalt additions significantly increased the yields of *Chrysochromulina polylepis* grown in Atlantic seawater 'spiked' with nitrate and phosphate (Granéli *et al*., 1993); this occurred

whether the cobalt addition was as the ion ($0.2–3 \times 10^{-9}$ M) or as vitamin B_{12} (Haraldsson and Granéli, 1995). Most seawater media have added cobalt as chelated Co^{2+} in the rather narrow range 10^{-7} M to 10^{-8} M and vitamin B_{12} at concentrations varying from almost 10^{-5} M to 10^{-10} M. The requirements for B_{12} in culture are very small; minimum cell quotas are from perhaps 10^{-20} to 10^{-16} mol cell^{-1} and half-saturation constants for growth range from 10^{-13} M to 10^{-12} M (Swift, 1980). Insofar as dilution of the other vitamins is concerned, the only guides are that, for thiamine-limited *Pavlova lutheri*, cell quotas and growth half-saturation concentrations were 10^{-19} mol cell^{-1} and 4×10^{-10} M. For biotin-limited growth of the dinoflagellate *Amphidinium carterae* these values were respectively 1.8×10^{-19} mol cell^{-1} and 1.6×10^{-11} M (Carlucci and Silbenagel, 1969).

The nature and amount of chelator used in enrichment cultures can have extremely selective effects. The role played by the chelator is to maintain or create a favourable trace-metal environment for the cells (Sunda, 1989). In the case of most trace metals this is accomplished by the buffering action of the added chelator-trace metal mix on the concentrations of the free metal ions, or of inorganic ionic species proportional to them. The case of iron differs significantly in that the biologically active ionic iron species derive only in small part or indirectly from dissociation of the Fe (III)-chelator complex according to the thermodynamic equilibria. They must derive from photochemical reactions in which the chelator is oxidized and decomposed while the iron is reduced to Fe (II), from which usable Fe (III) species or colloids are rapidly formed (Price *et al*., 1989; Wells, 1989). The chelators most often employed are EDTA, NTA (nitrilotriacetic acid), or citric acid, all usually specified as the sodium salts. EDTA is the strongest complexer and the chelator most commonly employed, NTA is less commonly used, while citric acid is the weakest and has a longer history of use than the other two. Empirically it has been found that the addition of citrate to enrichment cultures (or even growth media) having other chelators favours some organisms. Citrate, the weaker chelater, equilibrates much more rapidly with metals than do the stronger chelators, which dominate the metal speciation ultimately. On the other hand, citrate ion forms complexes that can be transported into cells, carrying with it the complexed metal ions (Errécalde and Campbell, 2000).

Initial experience with the difficult *Aureococcus anophagefferens* is relevant. It was obtained into unialgal culture in sterile filtered local seawater enriched as f/2 medium (see Table 3.1) with glycerol phosphate replacing inorganic phosphate (Cosper *et al*., 1987). Yet, in a commercial synthetic seawater base ('Instant Ocean') it was necessary to use either NTA or citrate rather than EDTA (at equal molar concentrations) to obtain reasonable growth, even with glycerol phosphate replacing inorganic phosphate (Dzurica *et al*., 1989). Further, Cosper *et al*. (1993) found that citrate was the most effective chelator when used in chemically defined variations of medium Aquil. *Aureococcus* strains now in culture grow very well in L1 enriched seawater (Table 3.1). For this organism, ammonium has been found to be toxic at relatively low levels (Guillard, unpublished).

3.3.4 Processing enrichment cultures

Observation of all enrichment cultures should begin as soon as 24 hours after enrichment and continue daily because population changes can be very rapid. Direct isolation of cells by micropipette (Fig. 3.2E) is the method of choice for large single-celled

species whether motile or not and generally necessary for colonial forms such as *Phaeocystis, Trichodesmium* or chain forming diatoms or dinoflagellates. The general process has been described by Guillard (1973), Hoshaw and Rosowski (1973) and Guillard and Keller (1984). Cleaned glassware, including depression and other slides, is best sterilized in an oven (2 hours at 160°C) in order to be particle-free. First, place a well or depression slide on the stereo microscope stage using transmitted illumination, and add sterile particle-free 'washing' fluid (seawater or medium) to the wells with a sterile Pasteur pipette; add a drop of culture to the first well. If there are too many cells, move a drop from it to the next well, and repeat until algal units are separated enough to facilitate capture of one or only a few cells. Locate the algal unit desired under the lowest usable magnification. Before attempting to isolate it by capillary action into a drawn Pasteur pipette, touch the end of the capillary into a nearby well (drop) of sterile medium to draw a little liquid *(c.* 1 cm) into it as a 'cushion'. After a cell has been isolated, gently expel the captured cell (without the cushion) into a tube of sterile medium. Flagellate cells that show a strong phototactic response can be concentrated and often freed of other species by allowing them to swim in response to light across a Petri dish or other flat vessel containing sterile medium (see Guillard (1973) for Paasche's device for phototactic isolation).

3.4 Manipulations – antibiotics

Three somewhat different approaches of antibiotic treatment are described here (Fig. 3.2F). The first, 'hunt and wash' method (see Guillard, 1973), makes antibiotics an adjunct to micropipette isolation. A moderate inoculum of healthy and rapidly growing algal culture is transferred to fresh medium (50 ml in 125 ml flasks) containing a tolerable level of antibiotics and a small amount (*c.* one drop) of bacterial test medium to stimulate bacterial growth and thus susceptibility to the antibiotics. At several convenient times from 18 hours to 72 hours after exposure, one or a small number of cells is transferred by micropipette to each of several tubes of fresh medium (without antibiotics). There is a good chance that some tubes will then be bacteria-free (or at worst have fewer types of bacterial contaminants). The basic antibiotic solution recommended for this purpose is made as follows: dissolve 100 mg penicillin G (Na or K salt), 25 mg dihydrostreptomycin sulfate, and 25 mg gentamycin in 10 ml distilled (or deionized) water and sterilize by membrane filtration. Keep frozen until used (polycarbonate tubes are best). The 'standard' dose is 0.5 ml per 50 ml of medium; try 0.25 ml, 0.5 ml, 1 ml and 2 ml per flask.

The basic idea of the second method (Droop, 1967) is to dilute a strong antibiotic mix (made up in algal growth medium) using a dense but still rapidly growing algal culture as diluent. Do this in two-fold dilution steps so that the concentration of antibiotics is halved at each step while the concentration of algae is constant. As before, add a small amount of organic matter at the start. Very small transfers are made from each tube of the series at 24 hours and 48 hours into separate tubes of medium without antibiotics, and these are incubated. At some point in the decreasing series of antibiotics the algae are (hopefully) still alive while the bacteria are dead. Droop (1967) used mixes of four antibiotics. Berland and Maestrini (1969) provide excellent quantitative data on the effects of 25 antibiotics on five diatoms.

A third approach is sequential transfer of the algal culture through a series of flasks of media containing different antibiotics at levels permitting algal growth. The

aim is to lose the bacteria by attrition. Cottrell and Suttle (1993) thus purified the tiny flagellate species *Micromonas pusilla* using the following sequence of antibiotics: penicillin, 1 g l^{-1}; neomycin, 250 mg l^{-1}; gentamycin, 1 g l^{-1}; kanamycin, 0.5 or 1 g l^{-1}. The timing of exposure was critical.

No realistic recommendations can currently be given regarding choice of antibiotic treatments for purifying marine harmful algal species. If forced to make a choice among available antibiotics for single or multiple use, a reasonable combination at present might include: (a) penicillin G, ampicillin, or the much more expensive carbenicillin; (b) cephaloridine, which is moderately expensive, or cefotaxime; (c) gentamycin, about the same cost; (d) kanamycin, streptomycin, or neomycin – all inexpensive; (e) bacitracin, likewise inexpensive. Recent experience suggests that the macrolide antibiotic erythromycin will prove useful. This antibiotic should be dissolved in ethanol or 1–2 M HCl to make concentrated stock solutions.

An axenic culture in usual practice means 'without demonstrable unwanted prokaryotes or eukaryotes'. Demonstrations ordinarily include microscopic examination and inoculation from the culture under test into enrichment media. The basic sterility test medium now suggested, enrichment L1 pm (derived from its predecessor f/2 p), is given in Table 3.4. Tubes (or plates of medium solidified with agar, if used) should be examined frequently beginning about 48 hours after inoculation. Most bacteria develop within two weeks, but cold water forms and methylaminotrophs may take up to one month. Test media should be used in conjunction with fluorescence microscopy to verify purity. Fluorescent stains for revealing the presence of bacteria include DAPI and congo green.

TABLE 3.4 Sterility test medium L1 pm with methylamine added

Algal enrichment L1[a]	1 l
Bactopeptone[b]	1 g
Methylamine HCl[c]	0.68 g

Dissolve the peptone and dispense medium in 5–7 ml lots in tubes for testing algal cultures and in screw-capped glass or polycarbonate bottles for larger volumes.

a. seawater medium L1 according to Table 3.1. Dilute (with seawater) to 1/2 or 1/4 strength if desired. Keep Fe and EDTA about 10^{-6} M in any dilution.
b. Bactopeptone, neopeptone, trypticase and hycase do not precipitate in seawater; proteose peptone and tryptone do form precipitates in seawater. Test other commercial preparations. If it is necessary to use ones that precipitate, then an additional possibility is to autoclave them separately in distilled water, add aseptically to the rest and dispense aseptically.
c. This amount provides methylamine HCl (FW 67.52) at 10^{-2} M. It is convenient to make a molar stock solution in distilled water (6.75 g per 100 ml), autoclave in a screw-capped bottle and use 10 ml l^{-1} of L1 pm test solution before final autoclaving.

Source, Sieburth and Keller (1984).

3.5 MAINTENANCE OF STOCKS AND PRODUCTION

Success in maintaining stock cultures demands an unfailing year-round routine of preparing fresh medium and making transfers to it from the initial 'parent' culture, using an inoculum of appropriate size. It is advantageous and usually possible to schedule rapidly growing species for transfer once a week, slower growing ones at

two-week intervals, and many larger and slow growing algae (some dinoflagellates or chloromonads) at three-week intervals. The most important point is to transfer before half the total lifespan of a culture has passed. A culture transferred on a weekly basis should last one, or preferably two, more weeks if moved to dim light. It is often possible to keep both 'parent' and 'grandparent' cultures for security in case of equipment failure or other accidents (see Guillard, 1975 or 1983, for details).

The importance of unfailingly rigorous microbiological technique applied to all aspects of the transfer process cannot be overstressed. Procedures should be 'fail-safe'. This applies to such matters as accuracy in labelling tubes and flasks; ensuring that pipettes and media are sterile; having extra flasks or tubes to allow for spills, etc. Operators should practice aseptic techniques until skilled. Vessels are only open for a few seconds and are held so that nothing passes over the unopened tube or flask. An uninoculated tube or flask of organic medium (L1 pm) can be transferred serially to similar medium at one- to two-week intervals to check sterile techniques.

3.6 Media for maintenance of autotrophic cultures

The media used for continued culture of a strain after isolation can be either enriched natural seawater or enriched artificial seawater. In either case the ideas that emerged in the sections on general and selective enrichment media apply in significant ways. They may in fact apply also to cultures received from other laboratories or culture collections, because optimal conditions for culturing in larger volumes or for increased biomass may not have been worked out. Assuming that reasonably favourable conditions of salinity, temperature, and light regime have been found, the major choices in selection of a medium and of its sterilization in various volumes are: (a) chelation level; (b) ammonium concentration; (c) sterilization before or after enrichment. These are discussed below.

Concerning the first matter, that of chelation levels, a study of the hundreds of recipes that have been published for enriched natural or artificial seawater reveals two things: first, remarkable similarity, suggesting convergence on a generally acceptable formulation, and second, one difference that can be taken as significant – the orders of magnitude difference in the level of EDTA. Enrichments GPM (Loeblich, 1975) and K (Keller *et al.*, 1987) have EDTA at 10^{-4} M, while IMR (Eppley *et al.*, 1967) f/2 (Guillard, 1975; 1983) ESNW (Harrison *et al.*, 1980) and BWM (Brand, 1986) all employ EDTA at approximately 10^{-5} M. EDTA levels as low as 10^{-7} have been used successfully for culture of *Trichodesmium* sp. (Chen *et al.*, 1996). While high EDTA levels can lead to trace metal deficiencies, they may be useful in some cases such as that of *Chattonella ovata*. This species can be maintained at 10^{-5} M EDTA but grows better at 10^{-4} and can even tolerate 10^{-3} M. At the higher EDTA levels, this species does not adhere to a tenacious layer at the bottom of flasks, grows faster and attains a greater density.

The second factor in the choice of a medium is the ammonium concentration. Some algae that lack nitrate reductase, as do many cryptophytes, euglenoides and green algae, have an absolute requirement for nitrogen in the reduced state and can tolerate relatively high concentrations. However, the addition of ammonium must be used with caution because of the toxicity of free ammonia. Note that a seawater medium with ammonium can lose as much as 30% of the initial concentration

because of the high pH attained and the elevated temperature during autoclaving (Guillard, 1983). Keller *et al.* (1987) added 5 X 10^{-5} M ammonium chloride to K enrichment before autoclaving but cautioned that several algal strains could tolerate only half of this concentration even though some had an absolute requirement for ammonium.

Enriched seawater formulations with or without ammonium may be too concentrated for some species when they are first brought into culture. For initial enrichments of seawater or initial isolation of new cultures, ammonium should be reduced further in concentration or replaced partially or entirely by urea. Increase nutrient concentrations gradually in successive cultures. It is usually best to make a diluted medium by mixing sterile seawater with a previously autoclaved full strength medium, rather than by autoclaving a reduced medium, because of the loss of iron when the EDTA concentration is reduced below 10^{-6} M. Using the above method, the authors have successfully obtained isolates of *Gymnodinium breve* using f/10 (i.e. a 5-fold dilution of autoclaved f/2). Note that the morphology of cells in culture may change with the degree of enrichment. For example *Phaeocystis* will only remain in colonial form if subcultured in a 10-fold diluted medium. *Ceratium ranipes* will lose the characteristic "finger" morphology at the end of its horns if grown in concentrated media, but will retain this morphology when the cultures are grown in f/50 (Brand and Guillard, unpublished).

Lastly, the method of sterilization is very important to successful culturing no matter what medium is used. The basic choices are autoclaving without pH control, autoclaving with pH control, sterile filtration, and microwave sterilization. For routine maintenance of stock cultures in tubes or flasks of up to 50 ml, autoclaving of the complete medium is recommended for convenience and to minimize the chances of contamination. Most successful recipes for media were developed and tested under these conditions.

Tables 3.1 to 3.4 give the recipes for two enriched natural seawater media suitable for most harmful algae. Enrichment L1 (Table 3.1) is an improved version of the commonly used f/2, while L2 (Table 3.3) is a more highly chelated enrichment derived from several media described in the literature. As a general rule, coastal planktonic species will grow in L1 or f/2 while benthic, epiphytic, and oceanic planktonic species will grow in L2 or K.

The procedures described here and in the preceding section suffice generally for cultures of about 1 l. Good yields can be obtained if flasks are swirled and shaken gently once or twice per day. The supply of CO_2 will become growth-rate limiting at some point and usually crop limiting also as the culture pH rises. Bubbling with air or air slightly enriched with CO_2 (<1%) is adequate in cultures of this size. Some organisms tolerate violent bubbling, especially when cultures are dense, but others, such as some dinoflagellates, are sensitive and bubbling should be moderate and along the edge of the flask. Bubbling also helps cells in dense cultures to circulate throughout the illuminated regions of the culture vessel.

Autoclaving volumes of greater than 1 l has drastic effects which must be taken into account. The main factor is the high pH attained when CO_2 is expelled from the liquid during autoclaving; the time of exposure to this high pH must be longer because of the time needed to sterilize larger volumes. Further, equilibrium with atmospheric CO_2 is slower because of the larger mass of water and the smaller surface to volume ratio. Precipitates are formed, altering both the physical environments for the algal cells and the chemical composition of the medium.

Concerning the former, many flagellates, especially dinoflagellates, become entangled in precipitates and settle with them, or migrate downwards into them; some coccoid species become clumped onto sediment particles. In either case an inoculum can fail or be reduced effectively. Chemically, even unenriched seawater autoclaved in glass can yield precipitates containing minor elements (Jones, 1967) and dissolved silicic acid, as much as 290 µmol l^{1} (Guillard, 1983). Enriched seawater media such as f/2 form precipitates containing much of the added iron and phosphate (Harrison *et al*., 1980).

Certain treatments can reduce the problems of precipitates in large volumes of autoclaved seawater. Sterile nutrients can be added after the seawater is cooled and equilibrated with the atmosphere. Lowering the pH to around 4 by mineral acid or by bubbling with CO_2 before autoclaving can prevent the formation of precipitation. After autoclaving with acid (HCl or H_2SO_4) the pH can be returned to the original by the addition of sterile NaOH (see Harrison *et al*., 1980). Lowering the salinity helps, but it must remain in the range favourable to the species being cultured.

Many successful media, especially synthetic ones, use organic buffers to stabilize the pH during autoclaving. TRIS (hydroxymethyl amino methane) is the best known and least costly. It can be prepared as a stock solution of the pH desired and added to a final concentration of 1–5 µM. Note however that some algae, especially dinoflagellates, grow more slowly if TRIS is added to a successful medium.

3.6.1 Artificial seawaters

Recipes for artificial seawaters, sometime referred to as 'defined media', have also been introduced in great numbers. Synthetic seawaters are of two types. The first type, imitations of natural seawater, includes Aquil (Price *et al*., 1989) and ESAW (Harrison *et al*., 1980). The contents of both are compared by Brand (1990, Table 4). The second type differs from natural seawater in ionic ratios. Examples include the ASM and ASP series (Provasoli *et al*., 1957) as well as several formulations by Droop and others (Droop, 1969). The significant differences lie in the minor elements, chelators and organic additions.

For the use of artificial seawaters, the following approach is suggested. First, make an appropriate mix of the major and minor conservative elements. Table 3.5 is a suggested formulation. Next, enrich the synthetic seawater with N, P, Si, vitamins and trace metals as for natural seawater (e.g. as L1 or L2) and sterilize as appropriate, by autoclaving, filtering or microwaving.

3.6.2 Synthetic seawater

Many manufacturers exist for commercially available synthetic seawaters. These include Marinemix, Seachem Reef Salt, Kent Sea Salt, Coralife Salt, Instant Ocean, and synthetic f/2 by Sigma. These synthetic seawaters can be used as above for artificial seawater formulations. Note that some of them have added nutrients.

3.7 Methods for heterotrophic cultures

Heterotrophic forms, especially dinoflagellates, have become important HAB species in recent years. Two heterotrophic nutritional types can be found: osmotrophs and phagotrophs. Osmotrophs utilize dissolved organic matter, while phagotrophs feed on particulate organic matter. Guillard and Keller (1984) summarized defined

TABLE 3.5 Artificial seawater base AK

Compound	Concentration (mol l^{-1})	Weight per litre
NaCl	4.00×10^{-1}	24.00 g
KCl	8.00×10^{-3}	0.60 g
$MgCl_2$ $6H_2O$[a]	1.48×10^{-2}	3.00 g
$MgSO_4$ $7H_2O$	2.03×10^{-2}	5.00 g
$CaCl_2$ $2H_2O$	2.70×10^{-3}	0.40 g
NaBr	1×10^{-5}	1.03 mg
H_3BO_3[b]	9.70×10^{-5}	6.0 mg
$SrCl_2$ $6H_2O$	1×10^{-4}	26.6 mg
NaF	1×10^{-5}	0.42 mg
KI	2.00×10^{-7}	33.00 µg
$NaHCO_3$[c]	2.38×10^{-3}	0.20 g

a. Dissolve the hydrated salts separately in distilled or deionized water and add to the other compounds (except for $NaHCO_3$) already mixed.
b. Ten times the concentration in AK.
c. Keller *et al.* (1987) added the $NaHCO_3$ just before autoclaving and adjusted the pH to 7.0 with HCl. After autoclaving and equilibrating with air, the pH returned to c. 8.0. This lowers the alkalinity by an indeterminate amount. If this is undesirable for some reason, the $NaHCO_3$ can be added and the pH lowered with bubbled CO_2 as in the preparation of Aquil, or by adding additional equimolar amounts of $NaHCO_3$ and HCl, as in the preparation of ESAW.

Source: modified from Keller *et al*. (1987).

media used for the culture of osmotrophs. Some phagotrophic dinoflagellates, such as *Crypthecodinium cohnii*, *Oxyrrhis marina*, and *Noctiluca scintillans*, are capable of growing osmotrophically. Other heterotrophic dinoflagellates, such as *Pfiesteria piscicida* and *Protoperidinium crassipes*, are obligate phagotrophs.

Procedures to obtain cultures of heterotrophic forms are the same as for autotrophic ones except that a food source is required. Determining the optimum food organism and essential nutrient requirement for each heterotroph is a mixture of trial and error and knowledge of nutrient requirements of other protists (Gaines and Elbrachter, 1987). Some generalizations can be made, however. Large athecate dinoflagellates can be cultured using smaller dinoflagellates such as *Scrippsiella* and *Gymnodinium* as food, while large thecate dinoflagellates can be cultured on both dinoflagellates and diatoms (Jacobson and Anderson, 1987). A mixture of food sources rather than a single species often results in better growth (Lessard, 1993). Small athecate and thecate heterotrophic dinoflagellates can use small flagellates and cyanobacteria as a food source. An extreme example is *Pfiesteria piscicida*, which will feed on bacteria, cyanobacteria, diatoms, dinoflagellates, raphidophytes or other small autotrophs (Burkholder *et al*., 1989).

The nutritional requirements for most phagotrophic dinoflagellates have not been fully studied; growth of most can be enhanced by the addition of vitamins and trace metals. Gifford (1985) found that growth of marine ciliates increased when grown in f/10 rather than unenriched seawater. Lessard (1993) used added K trace

metals (Keller *et al*., 1987) and vitamins to achieve maximal growth of several phagotrophic dinoflagellates.

Two different approaches for the culture of phagotrophic forms have proven successful: the 'frequent-feeding' method and the 'co-transfer' method. In the frequent-feeding method, small portions of food culture are added approximately once a week. The heterotrophic dinoflagellate culture is transferred to fresh sterile seawater along with any remaining prey and re-fed as before. This method seems to work well with most heterotrophic forms. In the co-transfer method, the heterotrophic dinoflagellate and prey organism(s) are transferred into a complete enriched medium such as f/2 or L1. The heterotroph and the prey organism grow together in a relatively stable association. This method can have the problem of the prey organism overgrowing the heterotrophic organism. However, diminishing the light level can control the autotrophic prey density. Many small athecate dinoflagellates such as *Oxyrrhis marina* can be cultured by this means, as can the larger *Noctiluca*.

3.8 Mass culture

For the purification of new toxins, production of toxin standards, and other experiments, large amounts of cultured material are required. Three distinct approaches to mass culture are available: continuous, semi-continuous, and batch. Continuous culture systems, either turbidostats or chemostats, have been reviewed in depth by Droop (1975). With the continuous-culture approach the cell number (turbidostat) or chemical composition of the culture medium (chemostat) is kept constant. A continuous culture can be kept in log phase growth. A semi-continuous culture is allowed to reach a specific point in the growth phase then a portion of the culture is harvested. New medium is then added to allow continued growth back to the harvested cell concentration. The batch mass-culture method allows the cultured population to go though the normal growth stages of lag, log and senescence. The culture is harvested at any point of the growth phase, usually at the end of the log phase of growth. With the batch-culture method there is little or no chance of the introduction or accumulation of a contaminant.

Stock cultures kept in tubes or small flasks require a 'scale-up' phase regardless of which type of mass-culture technique is employed. As a general rule for harmful species, a 1/10 dilution of culture inoculum will prevent a long lag phase due to small inoculum size. For example, 125 ml of an exponentially growing stock culture is inocululated into a 1 l culture flask. Once this culture reaches late-log growth phase, this culture is used to inoculate a 10 l carboy. This is continued until the desired culture volume is reached. A simple and inexpensive carboy system is shown in Guillard (1983). Note that for some rapidly growing autotrophs, such as small raphidophytes and prymnesiophytes, a 1/100 dilution can work.

The production of dense cultures of organisms that require reduced nitrogen, such as *Aureoumbra lagunensis*, poses special problems. The total ammonium required for a dense crop cannot be added at the start of a batch culture because ammonium (as ammonia) becomes toxic (to most species) at levels well below the amount needed for a dense crop. Ammonium must be added at intervals throughout the growth phase at a non-toxic concentration. A similar stepwise addition of silicate is required for dense cultures of toxic diatoms.

Once the desired volume of culture is reached, many factors need to be taken into account to achieve maximum cell densities. Light levels must be increased for

autotrophs as cultures become dense. Dense cultures of autotrophic organisms commonly raise the pH to as high as pH 10 by removing CO_2 from the water. At these high pH values growth and cell densities can be reduced. Aeration with air alone or air enriched with CO_2 can control the pH. Cultures of heterotrophic organisms may require the addition of O_2 to allow for respiration; aeration provides oxygen and removes the excess CO_2 produced during respiration.

However, aeration of cultures should only take place if the chemical nature of the toxin is known. For example, *Karenia brevis*, which produces brevetoxin, is known to lyse, releasing its toxins as an aerosol. This aerosolized toxin can cause brevetoxicosis to laboratory workers if breathed. It cannot be stressed enough that mass culture of algae with unknown toxins which may produce aerosol or volatile compounds should not be aerated unless precautions are taken. The most drastic example is the mass culture of *Pfiesteria piscicida* in the presence of fish. These organisms must only be cultured in a bio-safety level III (BSLIII) facility as the putative toxin produced by this dinoflagellate is known to cause severe adverse symptoms in laboratory personnel exposed to aerated cultures.

Another factor in mass culture is the reduction of self-shading. The advent of suspension culture eliminates both factors. Fig. 3.3 outlines a suspension-culture

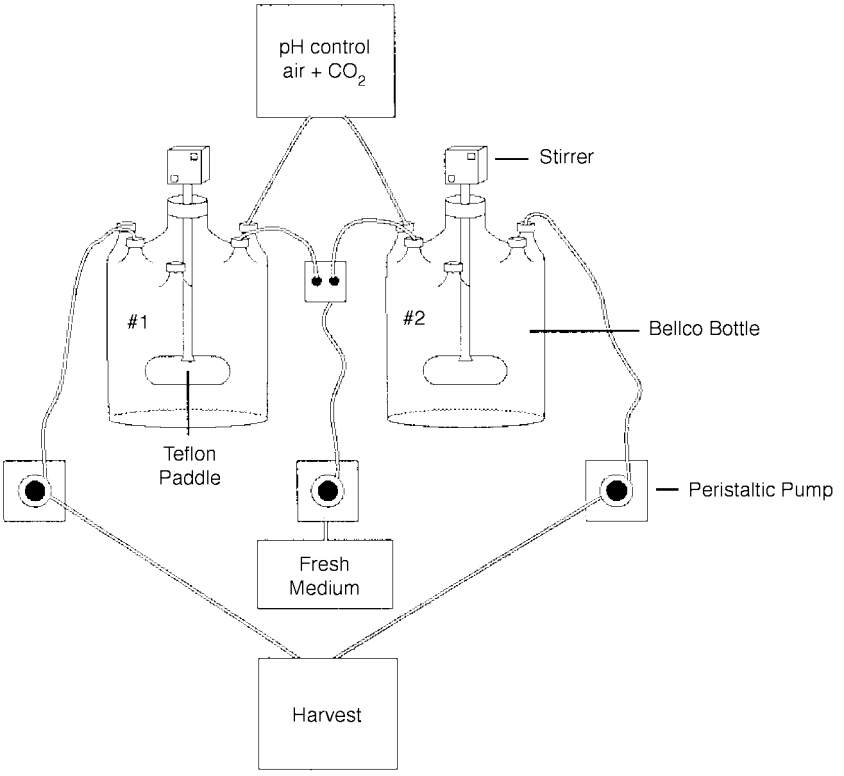

Figure 3.3
Large-scale suspension culture apparatus with pH control (after Morton and Bomber, 1994).

approach used in the culture of *Prorocentrum hoffmannianum* (Morton and Bomber, 1994). Increased biomass was achieved by utilizing both CO_2 control and slow stirring of the culture using Teflon paddle impellers. The authors have successfully mass-cultured both planktonic and benthic algae using this method. The speed at which the paddle impellers spin is critical. Generally, planktonic forms are more sensitive than benthic forms. Thus, benthic forms can be spun at a greater speed than planktonic forms.

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4

Estimating cell numbers

P. Andersen and J. Throndsen

4.1 Introduction

Quantification of microalgae is one of the basic routines in HAB monitoring programmes or research projects. Qualitative investigations will reveal which species to take into account, but when assessing risks precise knowledge on the abundance of the harmful species is essential. In general, high concentrations of harmful algae indicate a high risk of harmful effects, whereas the same species may appear in low concentrations with no harmful effects. The harmful concentration of a HAB organism is species-specific. Some algae are harmful at low concentrations, such as species from the genus *Alexandrium*, which may cause accumulation of PSP toxins in shellfish at concentrations of a few hundred cells per litre, whereas other species such as *Karenia* (*Gymnodinium) mikimotoi* can cause fish kills at millions of cells per litre (Zingone and Enevoldsen, 2000). This chapter discusses several microscope-based methods for quantification of microalgae at the species/genus level. Prior to that we present guidelines to sampling and preservation of samples. Furthermore we suggest a simple appropriate statistical routine to calculate the precision of counts as well as how to estimate microalgal biomass using geometric formulae and appropriate conversion factors. A culturing approach (serial dilution culture method) for the estimation of selected HAB species is also presented. If algal data are to be available for analysis, or to be compared with events elsewhere or in the past, it is important to have easy access to validated data. We discuss the use of databases and spreadsheets for data storage and handling as well as quality assurance measures to be taken.

4.2 Planktonic microalgae

4.2.1 Sampling

Qualitative, concentrated samples are best collected by vertically towing a plankton net (mesh size 20 µm) to cover the depth range of interest. The plankton net should be drawn, several times, to the surface of the water, until the water in the sample collector becomes coloured by the algae. Quantitative samples (water/bottle samples) can be collected using a water bottle (e.g. Niskin, Nansen) at different depths to cover the depth range of interest. Depth intervals between sampling should be 2–5 m, dependent on local conditions. The samples from the different depths can be pooled and counted as one sample representative of the whole water column. An alternative to sampling with water bottles is the use of a hose for sampling the whole water column as described by Lindahl (1986), for example.

4.3 FIXATION

Immediately after collection the samples must be preserved for later analysis in the laboratory. Microalgal samples should be preserved using either neutral or acidic Lugol's solution (Table 4.1), which produces good preparations for light microscopy and is of low toxicity to humans. If the brownish coloration of the algae, caused by Lugol's imposes a problem in taxonomic investigations, the coloration can be removed by oxidizing the Lugol's using a few drops of a solution of sodium thiosulfate per ml (3 g $Na_2S_2O_3$ for 100 ml of water) of sample (Pomeroy, 1984). Fixation of samples using Lugol's increases the settling velocity of microalgae compared with samples fixed in formaldehyde (Table 4.2).

TABLE 4.1 Recipes for Lugol's solution (acidic, alkaline or neutral)

Acidic	**Alkaline**	**Neutral**
20 g potassium iodide	20 g potassium iodide	20 g potassium iodide
10 g I_2	10 g I_2	10 g I_2
200 ml distilled water	200 ml distilled water	200 ml distilled water
20 g acetic acid	50 g sodium acetate	

Note: Add approximately 0.5–1.0 ml Lugol's/100 ml sample – the fixed sample must be brownish (the colour of brandy).

TABLE 4.2 Recipes for formaldehyde fixatives

Neutralized formaldehyde[a]	**Formaldehyde (non-neutralized)**[b]
500 ml 40% formaldehyde	100 ml 40% formaldehyde
500 ml distilled water	700 ml filtered seawater
100 g hexamethylenetetramine	

Note: pH 7.3 to 7.9
Filter after one week

Sources:
a. Edler (1979).
b. Andersen and Sørensen (1986).

Neutralized formaldehyde should be used with care in a fume hood, because of its toxicity (potential carcinogen), and its potential to develop allergic reactions in humans. Edler (1979) recommends the use of a 20% formaldehyde working solution and a final concentration of 5% or more. Andersen and Sørensen (1986) used a working solution of 5% and a final concentration of 1.5% (Table 4.2). Use of fresh formaldehyde that is less than six months old is recommended, especially if non-neutralized because this fixative becomes acidic over time.

4.4 STORAGE/MAINTENANCE OF FIXED SAMPLES

Keep the fixed samples in glass bottles in the dark to prevent the Lugol's from being degraded by oxygen and light. Check fixation each month and add new Lugol's to the samples if they turn 'clear' (non-brownish). Properly fixed samples can last for years.

4.5 Handling of samples

4.5.1 Concentration/dilution of samples

Prior to quantitative analysis it may be useful to either concentrate or dilute the samples to obtain concentrations that can be handled using available techniques. Samples can be concentrated by a factor of 10 to 100 by settling of the cells in a measuring cylinder. Alternatively, cells can be concentrated by gentle filtration using, for example, a plankton net (mesh size 10 µm or 20 µm) or a GFC filter with a vacuum pump. Furthermore, microalgae can also be concentrated using the gentle 'inverted filtration method' described in Sieburth (1979). Note that cells can be lost during the concentration procedure. In most cases it is preferable that algal samples are fixed before concentration. If the microalgae are too concentrated the samples can be diluted with filtered seawater.

4.5.2 Setting up samples for quantification

Samples must be adapted to room temperature to reduce the risk of air-bubble formation in counting cells/chambers. To make sure that the algae are evenly distributed in the sample, turn the bottle upside down some 100 times before subsamples are extracted from the bottle. In the case of counting colonial species such as the cyanobacterium *Microcystis*, the colonies can be fragmented into single cells using sonication for 15–60 s at 20–40 kHz before counting (Cronberg, 1980).

4.6 Counting techniques

Prior to quantitative analysis of harmful algae, qualitative analysis of concentrated plankton is a must to establish which species could be misinterpreted during quantification. The magnification used when counting the different species must be adapted to the size of the species of interest (Table 4.3). Normal light microscopy is useful in general. Phase-contrast light microscopy may be useful in the case of species with delicate structures such as spines, scales, flagella and lorica.

Table 4.3 Recommended microscope working magnifications for counting different-size classes of microalgae

Class	Magnification
0.2–2.0 µm (picoplankton)	1 000×
2.0–20.0 µm (nanoplankton)	100–400×
>20.0 µm (microplankton)	100×

Source: HELCOM (2000).

4.6.1 Choice of method

Microalgae can be quantified by a range of different methods based on compound microscopy, inverted microscopy or epifluorescence microscopy (Table 4.4).

TABLE 4.4 Summary of methods for quantification of harmful microalgae

Methods for quantification of microalgae	Volume (ml)	'Sensitivity' (cells l^{-1})	Preparation time
Compound microscopy			
Drops on slide	0.02	50 000–100 000	1 min
Sedgewick-Rafter cell (counting cell)	1	1 000	15 min
Palmer–Maloney cell (counting cell)	0.1	10 000	15 min
Inverted microscopy			
Utermöhl (sedimentation chamber)	2–50	20–500	2–24 hours
Epifluorescence microscopy			
Counting on filters (fluorochrome: Calco Fluor, DAPI, Acridine Orange, etc.)	1–100	10–1 000	15 min

Source: Andersen (1995).

4.6.2 Compound microscopy

A simple method of quickly estimating the concentration of microalgae is to use the 'drop on slide' method. One drop of seawater is approximately 20 µl or 0.02 ml. The drop is placed on a slide, mounted with a cover slip and counted at appropriate magnification to observe the species of interest. If you find one cell in the drop this is equal to approximately 50 cells ml^{-1} = 50,000 cells l^{-1}, see Table 4.4. This method is only useful to screen for algae in high concentrations.

With concentrations of harmful algae >10^4 cells l^{-1} counting using a compound microscope and a counting cell is simple and fast (Table 4.4). If, on the other hand, the cell concentration is <10^2–10^4 cells l^{-1}, the cells must be concentrated before counting. This is a time-consuming procedure. In this case counting using either inverted microscopy or epifluorescence microscopy is preferable (see below).

Counting cells are available in several different volumes (Fig. 4.1). A well-known type is the Sedgewick-Rafter cell, with a volume of 1 ml. The bottom plate of the Sedgewick-Rafter cell is divided into 1,000 squares, each representing 1/1,000 of the volume of the cell. Other types of counting cell hold different volumes (Table 4.4). You can make your own counting cells using a slide mounted with a 'frame' on top. The exact volume of the home-made counting cell may be estimated as the difference in weight between the empty counting cell (including the cover slip) and the filled counting cell (including the cover slip) carefully dried on the outside of the chamber using a tissue to remove excess water.

Using a counting cell you can choose either to count the microalgae in the entire bottom of the chamber or only a fraction of the entire bottom. Knowing the fraction of the entire bottom counted and the number of microalgae encountered, the total number of microalgae in the counting cell can be calculated. Remember to incorporate the dilution/concentration factor of the sample when calculating the concentration of the microalgae (cells l^{-1}).

4.6.3 Inverted microscopy

Quantification of harmful algae using inverted microscopy and sedimentation chambers according to Utermöhl (1958) is useful for counting algae in rather low

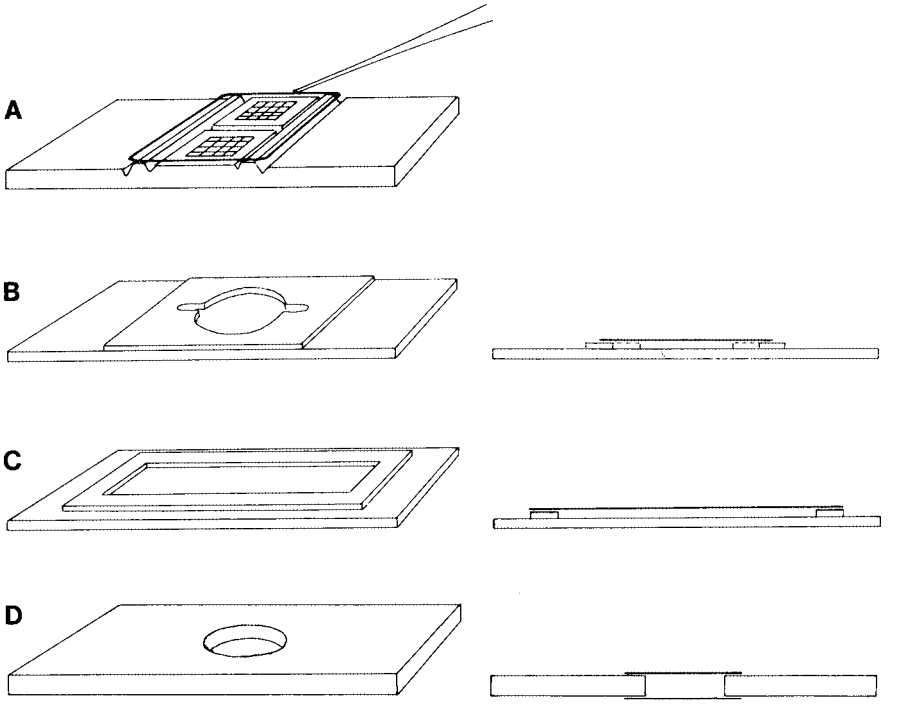

Figure 4.1
Counting cells.
Source: Throndsen (1995).

concentrations (<10^2–10^4 cells l^{-1}) (Sournia, 1978). If concentrations are higher the samples can be diluted using filtered seawater before counting. Equipped with suitable slide holders, an inverted microscope is also excellent for qualitative examination of normal slide preparations or quantitative analysis using counting cells. Sedimentation chambers are available from different companies in a range of volumes from 2.5–50 ml. In order to keep the sedimentation chambers tight, the bottom of the 50 ml settling cylinder may be greased with a thin layer of Vaseline before it is mounted on the plate chamber. Likewise, the top of the settling cylinder may be greased to keep the cover slip in place and to keep the settling cylinder properly sealed and airtight. During filling and sedimentation, the sedimentation chambers should be placed on a horizontal surface to promote a random distribution of the microalgae settling out. Furthermore, it is very important that the supporting surface is vibration-free, as vibration can cause cells to accumulate in ridges (HELCOM, 2000).

Settling of cells in the sedimentation chamber lasts from a few hours to several days depending on the sample volume (the height of the chamber), the fixative used and the linear dimension of cells. In general, small cells have much longer sedimentation times than large cells. As a rule large cells (L >10 µm) must be allowed to settle for at least 12 hours before counting, while smaller cells must be allowed to settle for approximately 24 hours before counting (Table 4.5). Sedimented samples not

counted within a week should be discarded. Sedimentation chambers of 100 ml should be used with caution as convection currents have been reported to interfere with sedimentation of the microalgae in chambers taller than five times their diameter (Nauwerck, 1963; Hasle, 1978).

TABLE 4.5 Minimum sedimentation times for Lugol's fixed samples

Volume of sedimentation chamber (ml)	Approx. height of sedimentation chamber (cm)	Sedimentation time (h)	
		Lugol's (acidic)	Formaldehyde (neutral)
2	1	3	12
10	2	8	24
50	10	24	48

Source: adapted from Edler (1979).

The bottom of the sedimentation chamber is scanned at different magnifications and a preliminary species list is produced. If the distribution of microalgae on the bottom of the chamber is visually non-random, one-sided or in ridges due to factors such as vibration, the sedimentation chamber should be discarded. According to the size of the different species as well as the abundance of the species, a strategy for counting each of the species of interest is set up, including

- choice of magnification;
- the total bottom of the chamber or subsampling units (half the bottom, diagonal counting, counting grids, etc.) (Fig. 4.2);
- approximate number of subsamplings.

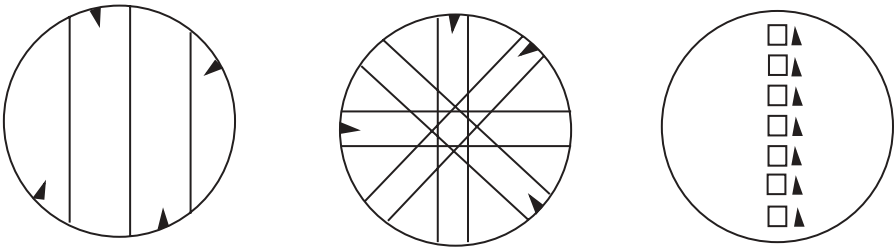

Figure 4.2
Counting strategies using sedimentation chambers. A, counting the entire bottom; B, counting diagonals; C, counting grids (the grid is mounted in one of the eyepieces).
Source: adapted from HELCOM (2000).

The goal of the counting strategy is to obtain a count of 50 to 100 cells of species of interest to assure a proper estimate of the concentration of each. Counting statistics are discussed below. If possible, the individual number of cells in colonial species should be counted. In the case of filamentous species without clearly differentiated cells, such as the genus *Nodularia* or *Trichodesmium*, the number of filaments is counted. In many cases the cells will not be randomly distributed on the bottom of the sedimentation chamber. The larger species often tend to accumulate at the edge of the chamber and the smaller species in the central part (Olrik *et al.*,

1998). Regardless of the counting strategy, it is important to compensate for non-random distribution either by counting diagonals, grids along a diagonal, or the entire bottom of the sedimentation chamber. When counting cells in a diagonal or within a grid you have to make a rule as to which cells are inside/outside the counting area. A simple rule could be that all cells inside or touching the left side of the diagonal are counted while cells outside or touching the right side of the diagonal are omitted from the count.

To calculate the concentration (cells l^{-1}) of the different species in the counting chamber you need to know

V = volume of chamber (ml);
B_a = area of bottom of chamber (mm^2);
B_c = area of part of bottom counted (mm^2);
N = number of cells scored for species of interest;
conversion factor (CF) = B_a/B_c.
The concentration C of species (cells ml^{-1}) is then
$C = N \times (B_a/B_c)/V$.

It is not possible to obtain general conversion factors to be used for all combinations of microscopes, counting magnifications and counting/sedimentation chambers when calculating concentrations from counts such as those done by diagonal counting. This is because the conversion factors are dependent on the magnification used as well as the type of sedimentation chamber. The conversion factors are to be calculated for each microscope and each subsampling area for each magnification, as well as for each type of sedimentation chamber (Table 4.6). Note that the dimensions of commercial chambers vary. It may be necessary to label and calibrate each chamber separately.

4.6.4 Quantitative epifluorescence microscopy

The basic principles of quantitative epifluorescence microscopy are concentration and staining of cells on membrane filters (Fig. 4.3), followed by quantification of cells using an epifluorescence microscope (Fig. 4.4). The method is useful for counting algae in low concentrations ($<10^2$–10^4 cells l^{-1}). If concentrations are high the samples can be diluted using filtered seawater. For thecate dinoflagellates the fluorochrome Calco Fluor White MR2, a specific stain for cellulose, is excellent (Lawrence and Triemer, 1985; Andersen and Kristensen, 1995). For quantification of harmful algae in general, other stains such as Acridine Orange (Andersen and Sørensen, 1986) or DAPI (Porter and Feig, 1980) can be very useful. A great advantage of this methodology is that large volumes (50–100 ml) can be prepared for quantification in a few minutes, and that specific stains, such as Calco Fluor White, allow for counting thecate dinoflagellates in low concentrations in situations where the overall cell concentration and/or the concentration of detritus is very high, because only the thecate dinoflagellates will 'light up' in the preparation.

A normal compound microscope can be transformed into an epifluorescence microscope if it is equipped with a halogen lamp/mercury burner and suitable filter sets for the stains used, see below.

Follow the procedure described in Sections 4.5 and 4.6. For quantitative epifluorescence microscopy the sample does not need to be adapted to room temperature because the formation of air bubbles is not a problem when preparations are made on filters.

The sample is prepared following this procedure:

1. Measure out the volume of sample to be counted using a measuring cylinder. Stains such as Acridine Orange or DAPI can be added directly to the sample at this point. If you use Calco Fluor White for staining thecate dinoflagellates, the stain must be added later, see steps 4 and 5.
2. Add the sample to the filtration unit (pore size of filter e.g. 5 µm). You can use other pore sizes depending on which species are to be concentrated and quantified.

TABLE 4.6 Example of calibration table used for calculating concentrations of microalgae by inverted microscopy and Utermöhl method (area of sedimentation chamber 531 mm^2)

	swi (small window)	**wi (window)**	**dwi (diagonal window)**
	L (mm) Area (mm^2)	*L* (mm) Area (mm^2)	Area (mm^2)
4×	0.202 0 0.040 8	2.02 4.08	 31.52
10×	0.081 0 0.006 6	0.81 0.66	 12.64
20×	0.040 0 0.001 6	0.40 0.16	 6.2
32×	0.020 0 0.000 4	0.20 0.04	 3.2
63×	0.008 0 0.000 06	0.08 0.006	 1.25
	CF: swi	CF: wi	CF: dwi
4×	13 014	130	16.9
10×	80 455	805	42.0
20×	331 875	3 319	85.7
32×	1 327 500	13 275	166.0
63×	8 850 000	88 500	425.0

Calculation of concentrations using the conversion table:
cells/ml = (cells subsampling $unit^{-1} \times$ CF)/volume of chamber.
Calculating concentrations:

Example 1:
Volume of chamber = 100 ml
Counts (entire bottom of chamber) = 50 *Dinophysis acuminata*
Calculating concentrations:
(50/100) = 0.5 cells ml^{-1} = 500 cells l^{-1}

Example 2:
Volume of chamber = 100 ml
Counts (dwi 10×) = 50 *Dinophysis acuminata*
Calculating concentrations:
(50 × 42.0)/100 = 21 cells ml^{-1} = 21,000 cells l^{-1}

3. Turn on the vacuum pump (maximum pressure 200 mm Hg).
4. If you use the stain Calco Fluor White, turn off the vacuum pump when there is approximately 1 ml left in the filtration chimney.
5. Add three to five drops of Calco Fluor working solution (concentration 2 mg l^{-1}).
6. Turn on the vacuum pump again and filter until the filter goes dry.
7. Take off the filter and gently dry the back on a tissue to eliminate surplus water.
8. Mount the filter on a drop of paraffin oil on a slide, add another drop of paraffin oil on top of the filter and put on the cover slip (24 × 24 mm).
9. Observe your preparation using an epifluorescence microscope with an appropriate filter setting for the fluorochrome in use.

4.6.5 Quantitative analysis of samples preserved in acidic Lugol's, formaldehyde or glutaraldehyde using the fluorochrome Calco Fluor

1. Measure out the volume of sample to work on in a measuring cylinder.
2. Adjust the pH of the sample to pH 7 using NaOH before you make your preparation or alternatively adjust the pH when you have completed your filtration procedure and have 1 ml left in the filtration chimney.
3. Add the sample to the filtration unit (pore size of filter e.g. 5 µm).

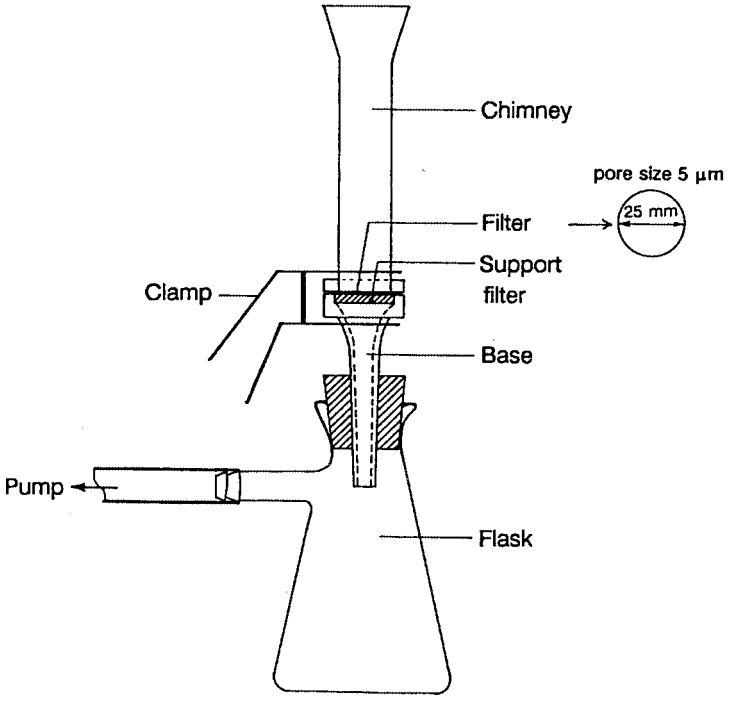

Figure 4.3
Filtration equipment used to concentrate microalgae on polycarbonate membrane filters for quantification using quantitative epifluorescence microscopy.

4. Turn on the vacuum pump (maximum pressure 200 mm Hg).
5. Turn off the vacuum pump when there is approximately 1 ml left in the filtration chimney (adjust pH if necessary – see step 2.).
6. Add three to four drops of Calco Fluor working solution (concentration 2 mg l^{-1}).
7. Turn on the vacuum pump again and filter until the filter goes dry.
8. Take off the filter and gently dry the back on a tissue to eliminate surplus water.
9. Mount the filter on a drop of paraffin oil on a slide, add another drop of paraffin oil on top of the filter and put on the cover slip (24 × 24 mm).
10. Observe your preparation using an epifluorescence microscope.

Note that Calco Fluor will only work at neutral pH (7), otherwise the Calco Fluor will precipitate – and you will see nothing but precipitated Calco Fluor!

The most frequent problems encountered when an epifluorescence method will not work are: (a) the filter set on the epifluorescence microscope does not work with the stain; (b) when using Calco Fluor, the pH of the sample to be analysed is not 7, or the working solution of Calco Fluor is too old.

Here are a couple of scenarios that you may encounter.

Problem # 1: You have made your preparation as described but see no fluorescence. What to do:

(a) Check that your mercury burner is working, if not, change the burner. If it still does not work contact a technician from the company that delivered the microscope.

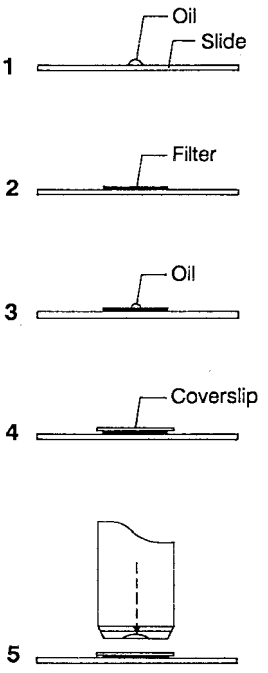

Figure 4.4
Mounting the polycarbonate filter on a slide for observation using quantitative epifluorescence microscopy.

(b) If the burner is working, make sure that you have the correct filter set and that the light from the mercury burner is not blocked and can be seen on the preparation. If the filter set is correct, but no light comes out of the objective, make sure that the light path is not blocked by other filters, etc.

Problem # 2: You have made a Calco Fluor preparation as described and see fluorescence all over the preparation and find it hard to localize the organisms. What to do:

(a) Check that the sample has a pH of 7. If the pH is not 7, adjust the pH as described and try again. If the pH is 7 then the Calco Fluor working solution might be too old. Make a new working solution and try again.

Counting procedure: start observing your preparation at a low magnification, for example using the 10× objective, as it is much easier to find some organisms to observe at low magnification! When you find some, make sure that the stain is working – that is, when using Calco Fluor White, the thecate dinoflagellates must light up blue on a dark background. If you use Acridine Orange the cells will light up orange, yellow and/or green on a dark background. If you use the stain DAPI the cells will light up blue on a dark background. When you have localized the organisms that you want to work on, switch to a higher magnification and adjust the intensity of the fluorescence using the different filters and screens mounted on the microscope.

The calculation is carried out basically as described for the Utermöhl method in Section 4.6.3. To calculate the concentration (cells l^{-1}) of the different species in the filtered sample you need to know:

V = volume of sample concentrated on filter (ml);
B_a = area of filter (mm^2);
B_c = area of part of filter counted (mm^2);
N = number of cells scored for species of interest;
conversion factor (CF) = B_a/B_c.
The concentration C of species (cells ml^{-1}) is then
$C = N \times (B_a/B_c)/V$.

As with the Utermöhl method, it is not possible to obtain general conversion factors to be used for all combinations of microscopes, counting magnifications and filters/filtering units to be used when calculating concentrations from counts such as those done by window counting. This is because the conversion factors are dependent on the magnification used when counting as well as the type of filtering unit. The conversion factors are to be calculated for each microscope and each subsampling area for each magnification, as well as for each type of filtering unit (Table 4.7).

4.7 Epiphytic/benthic microalgae

4.7.1 Epiphytic microalgae

Several methods have been used to sample and quantify epiphytic microalgae which cause ciguatera fish poisoning (Bagnis *et al*., 1980; Quod *et al*., 1995; McCafferey *et al*., 1992). According to Quod *et al*., epiphytic microalgae, including the

dinoflagellates responsible for ciguatera, can be sampled from macroalgae/sea grasses for quantitative analysis by the following procedure:

1. macroalgae are collected (20 g);
2. macroalgae are vigorously shaken in seawater (say for 30 s);
3. seawater is sieved (mesh size 150 µm);
4. dinoflagellates are investigated/quantified in the fraction <150 µm.

TABLE 4.7 Example of calibration table used for calculating concentrations of microalgae by epifluorescence microscopy (filter area 189 mm^2)

	swi (small window)	**wi (window)**	**dwi (diagonal window)**
	L (mm) Area (mm^2)	*L* (mm) Area (mm^2)	Area (mm^2)
4×	0.202 0 0.040 8	2.02 4.08	31.52
10×	0.081 0 0.006 6	0.81 0.66	12.64
20×	0.040 0 0.001 6	0.40 0.16	6.2
32×	0.020 0 0.000 4	0.20 0.04	3.2
63×	0.008 0 0.000 06	0.08 0.006	1.25
	CF: swi	CF: wi	CF: dwi
4×	4 632	46.3	6.04
10×	28 636	286	15.05
20×	118 125	1 181	30.50
32×	472 500	4 725	61.00
63×	2 953 125	29 531	152.00

Calculation of concentrations using the conversion table:
cells ml^{-1} = (cells subsampling $unit^{-1}$ × CF)/volume of chamber.
Calculating concentrations:

Example 1:
Volume of sample concentrated on the filter = 100 ml
Counts (entire filter area) = 50 *Dinophysis acuminata*
Calculating concentrations:
(50/100) = 0.5 cells ml^{-1} = 500 cells l^{-1}

Example 2:
Volume of sample concentrated on filter = 100 ml
Counts (dwi 10×) = 50 *Dinophysis acuminata*
Calculating concentrations:
(50 × 15.05)/100 = 7.5 cells ml^{-1} = 7,500 cells l^{-1}

The suspension containing the epiphytic species can then be investigated using light, epifluorescence or electron microscopy as described below. Cells can be 'extracted' from other substrates either by washing with filtered seawater, brushing or scraping. If the material fits under a microscope the surface can be investigated directly using epifluorescence microscopy or SEM.

A rough estimate of the abundance of epiphytic species can be obtained using counting cells, the Utermöhl method or epifluorescence microscopy according to Andersen and Kristensen (1995), see section 4.6.4. The abundance of epiphytic species can be presented as cells cm^{-2} or cells g^{-1} dw of substrate.

4.7.2 Benthic microalgae from sandy/muddy substrate

The substrate is sampled and either fixed in the field, using Lugol's or formaldehyde, or brought unfixed to the laboratory for extraction of live cells. Live samples must be kept in the dark, at *in situ* temperature and for as short a time as possible. Qualitative analysis can be carried out on live cells or cells fixed in Lugol's or formaldehyde using the same procedures as for plankton samples. The samples can be obtained using several different approaches. Species can be extracted from unfixed samples of sandy sediment samples using the Uhlig method (Uhlig, 1964), previously used for ciliates, for example (applying ice on top of the sediment kept in a cylinder with a mesh of 200 µm in the bottom which is in contact with water, e.g. in a Petri dish). In the case of muddy sediments, the mud can be spread out in a Petri dish and cover slips placed on the surface of the sediment – some species will then attach to the cover slip and can be investigated using light, epifluorescence or electron microscopy.

Finally the sediment can be spread in a thin layer on a slide and investigated directly. In the case of thecate dinoflagellates, the fluorochrome Calco Fluor (works only at pH 7; samples can be fixed in neutral Lugol's) can be added to the live samples or samples fixed in neutral Lugol's for investigation using epifluorescence microscopy.

Estimates of the abundance of benthic species can be obtained using counting cells, the Utermöhl method or epifluorescence microscopy according to Andersen and Kristensen (1995), see section 4.6.4. The abundance of benthic species can be presented as cells/cm^2 or cells/g dw of sediment.

Rough estimates of the abundance of species that can be cultured can be obtained from dilution series of sediment slurries using the most probable number (MPN) technique (see Section 4.12).

4.8 Statistics – how many cells to count

Counting microalgae can be a rather time-consuming process so it is preferable not to count too many cells to obtain a good estimate of the concentration in a sample. Under the assumption that the cells are randomly distributed in a sample, that is in the counting chamber or on the filter, the 95% confidence limits (Fig. 4.5) can be calculated according to the Poisson distribution:

$$= \pm(2 \times \sqrt{n} \times 100\%)/n = \pm 200\%/\sqrt{n}.$$

The more cells counted, the more precision gained (Table 4.8 and Fig. 4.5). At the beginning of the count, the precision is greatly increased each time you add

another cell. On the other hand, if you have already counted many cells only a small increase in precision will be achieved by adding more cells to the count. To obtain an estimate of the cell concentration with a precision of ±10%, you need to count approximately 400 cells. In many cases, where the species of interest occur in rather low concentrations (a few hundred cells l^{-1}), you may have to accept a precision of approximately 15–30%, corresponding to counts of 200 and 50 cells, respectively. In the case of colonial species, the input for the calculation of confidence limits must be the number of colonies counted and not the actual number of cells counted in the colonies. Note that the condition for the calculation of confidence limits, that the cells are randomly distributed, is not always met. In such cases the confidence limits calculated are too narrow (see Venrick, 1978, for further discussion of counting statistics).

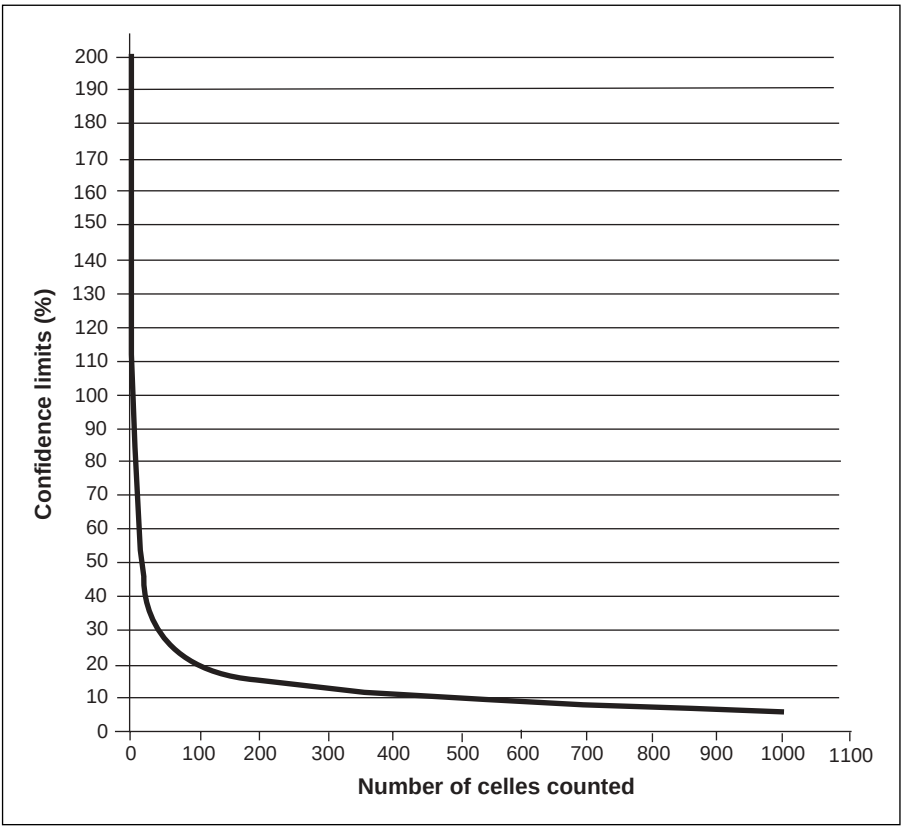

Figure 4.5
Relationship between number of cells counted and confidence limits (at 95% significance level).

TABLE 4.8 Relationship between number of cells counted and confidence limits (at 95% significance level)

Counts	Confidence limits ± (%)
1	200
2	140
4	100
5	90
10	63
20	45
40	32
50	28
100	20
200	14
400	10
500	9
1000	6

Example:
Sample volume = 100 ml
Counts = 50 *Dinophysis acuminata*
Concentration of Dinophysis:
50/100 = 0.5 cells ml^{-1} = 500 cells l^{-1}
Calculating statistics:
Relative limits of expectations:
±200%/√50 = 28%
Absolute limits of expectations:
28% of 500 cells l^{-1} = (500/100) × 28 = 140 cells l^{-1}
Final result:
Concentration = 500 ± 140 *Dinophysis acuminata* l^{-1}

4.9 ESTIMATING BIOMASS FROM MICROSCOPE OBSERVATIONS

Estimates of the biomass of microalgae can be very useful in many cases, such as to

- calculate the total phytoplankton biomass;
- calculate the biomass of taxonomical groups such as diatoms, dinoflagellates, etc.;
- calculate the biomass of functional groups such as auto-, mixo- or heterotrophs;
- compare the abundance of different species and their contribution to the total biomass of phytoplankton;
- evaluate to which extent one or several HAB species contribute to the pool of available food for filtering molluscs.

The biomass can be expressed as biovolume (mm^3 l^{-1}) which is approximately equal to the wet weight (mg l^{-1}) or as carbon biomass (µg Cl^{-1}). Biovolume can be calculated from measurements of linear dimensions of cells measured under the microscope using appropriate geometric formulae (Edler, 1979). Carbon biomass can be estimated from the biovolume using conversion factors from plasma volume

to carbon biomass. Edler (1979) suggests a conversion factor from plasma volume to carbon biomass of 0.11 for all phytoplankton and ciliates except thecate dinoflagellates. For thecate dinoflagellates a conversion factor of 0.13 is suggested.

4.10 Storage of raw data – data forms; spreadsheets; databases

All raw data should be filled into standard forms with information on

- position of sampling site;
- sampling depth;
- date;
- volume of sample used for quantitative analysis;
- method used for quantitative analysis;
- ID of person responsible for analysis.

Data should be stored as documentation for the calculated concentrations and biomass. Data can be stored in paper files, but preferably they should be stored in electronic form, either in spreadsheets or in a database. If raw data are properly stored it is possible to document the exact observations and to go back and recalculate concentrations and biomass of species if necessary. Furthermore, storage of data in databases facilitates data presentation/analysis including the plotting of population dynamics of selected species from week to week, or comparison of the abundance of microalgae between stations as well as between different seasons and years. Routines can be developed that make it very easy to present data stored in a database on the Internet , for example using Geographical Information System (GIS) technology. If samples of marine microalgae are routinely analysed, it is extremely helpful to use a database for storage of raw data as well as calculation of concentrations and biomass. Furthermore, the use of a database with a well-documented species list and basic information on how concentrations and biomass are calculated ensures that data are comparable for analysis and presentation. Use of such databases is implemented by several monitoring agencies, research departments and consulting companies worldwide.

4.11 Quality assurance

A detailed description of all procedures including the following should be produced:

- field sampling;
- handling of samples;
- qualitative analysis in the laboratory;
- quantitative analysis in the laboratory; and
- calculation of concentrations and biomasses.

Before the data are distributed throughout the monitoring system, they should be properly checked using a specified routine by at least one person who did not perform the quantitative analysis. Raw data should be kept on a long-term basis, in files for later documentation, examination or investigation.

4.12 Serial dilution culture (SDC) method

The present method (as introduced for phytoplankton by Knight-Jones, 1951, and later modified by Throndsen, 1978) has proved convenient, especially for the isolation of bloom-forming species. The principle of the method is to dilute the sample until only one specimen is left in the subsample (which is used as inoculum for cultures). Keeping track of the dilution, such as by proceeding stepwise, will also offer the opportunity to make estimates of the original cell number. By using three to ten parallel series of dilutions and applying statistics to the obtained pattern of presence and absence in each dilution, the probability of the estimate may be calculated. Presence and absence is revealed by growth or no growth in the tubes. To facilitate growth, the whole dilution series is made with a suitable medium (see Chapter 3). For toxic algal blooms, the quantitative estimates are often more easily made by direct counts but the SDC method will provide a convenient means for bringing the species into culture for further studies. The method has been applied with success to establish cultures of, for example, *Prymnesium*, *Chrysochromulina*, *Heterosigma*, *Aureococcus*, *Nannochloropsis* and single-celled stages of *Phaeocystis*. The SDC method can easily be combined with different media to find the most suitable one for a particular species under the defined laboratory conditions.

The dilution series could be achieved in two ways; the standard (pipette) dilution method based on separate dilution series set up for each parallel in the series, or the syringe method based on batch dilutions with subsamples inoculated into the parallels. The precision of both techniques will depend on the skill of the operator as well as the accuracy of the equipment. If the MPN is important, a test from algal material precounted, for example by haemacytometer (blood-counting cell), Coulter counter or flow cytometry, may be carried out to reveal deficiencies in the set-up or equipment (see concluding remarks). The pipette method requires one 10 ml graduated pipette for each parallel dilution series and culture tubes prefilled with exactly 9 ml of growth medium. The syringe method uses one 10 ml graduated syringe only, and the culture tubes may contain whatever amount of medium is convenient for the growth of the cultures. The syringe SDC technique may easily be adapted for inoculation into larger volumes of media and/or an increased number of tubes at each step (up to eight with the 10 ml syringe used as standard). Changing the volumes of the inocula or the size of the syringe used for the dilution will offer a variety of dilution possibilities. However, the mixing achieved in each type of syringe used has to be checked.

The following procedures for making a dilution culture series are carried out with simple equipment, and the initial steps (inoculation) can be performed in 5–10 min, on location. When on location take care to avoid direct sunlight and temperature shocks. The important precautions to be taken are to use clean equipment for culturing, to keep temperature and salinity well within the tolerance limits of the species, and to choose the appropriate medium. The fulfilment of the latter condition may be a matter of trial and error, but some media are more universal than others (e.g. Erd-Schreiber). Before deciding on the media to be used, consult Chapter 3. Another important measure to provide optimal culturing conditions is to prepare the medium from water sampled together with the inoculum. The rationale is that the organisms in question are adapted to this milieu; that possible hostile fellow organisms will be killed during the heating of the medium; and that nutrient depletion, which may be substantial at least under late bloom conditions, will be compensated

by the addition of nutrients to the growth medium. Filtration may not be a feasible method for sterilization of the seawater for the medium as viruses passing the filter may harm the cultures. Material to be used for starting SDC should be collected with a non-toxic water sampler and handled carefully to avoid temperature, salinity and light shocks. Before starting inoculation, ensure that the temperature and salinity conditions are the same in the sample and the medium, and that all equipment and accessories needed are at hand to facilitate rapid completion of the procedure. Tubes, pipettes and syringes which are presterilized at purchase ought to be tested for toxicity with sensitive algal species (e.g. *Chrysochromulina* sp.). Materials tolerated by the human organism may prove fatal to many algal cells.

4.12.1 Set-up for the syringe method

For a standard SDC series use a test tube rack that holds 30 (or more conveniently 40 to 50) test tubes in rows of five. Fill 25 test tubes with 9–10 ml of growth medium each, and mark the tubes for five dilution steps of five parallels, for example 1I, 1II, 1III (meaning first dilution step, parallel one, two and three), 2I, 2II, 2III, 3I, –, to 5I, –, at which step the inoculum will be 0.1 µl. Five tubes for performing the dilutions (Fig. 4.6D) are filled completely (15–20 ml). A disposable 10 ml sterile syringe, preferably with exocentric opening, and graduated into divisions of 1 ml, is also necessary. The amount of medium required for each SDC series is approximately 300 ml, but it may be wise to prepare extra in order to have a suitable medium for possible subculturing of interesting species that grow in the SDC.

Procedure. Start by rinsing the syringe with sample water, then (Fig. 4.6):

A, draw 10 ml of the water sample into the syringe;

B, dispense 1 ml into five of the first tubes (parallels I-V);

C, dispose of all but the last 1 ml from the syringe;

D, draw 9 ml of medium from one of the full tubes;

E, dispose of 1 ml.

Repeat steps B to E (Figs. 4.6F, 4.6G) for the next four dilution steps.

4.12.2 Set-up for the pipette method

For the pipette method, arrange 25 marked tubes in a rack, each filled with exactly 9 ml of culture medium. Have six culture-clean 10 ml graduated pipettes at hand. Total amount of medium required is 225 ml.

Procedure. Start with one graduated pipette (Fig. 4.7):

A, draw at least 6 ml of sample into the pipette;

B, dispense 1 ml into each of the five parallel tubes of the first step in the series;

C, with a new pipette, thoroughly mix the content of the first tube by sucking and ejecting;

D, draw 1 ml of suspension into the pipette;

E, add the 1 ml to the next tube in the dilution series;

F, with the same pipette, repeat mixing and transfer (C to E) for the remaining dilution steps.

Then, using each of the tubes filled in step B as basis, repeat the dilution procedure (C to F) for each parallel in the series.

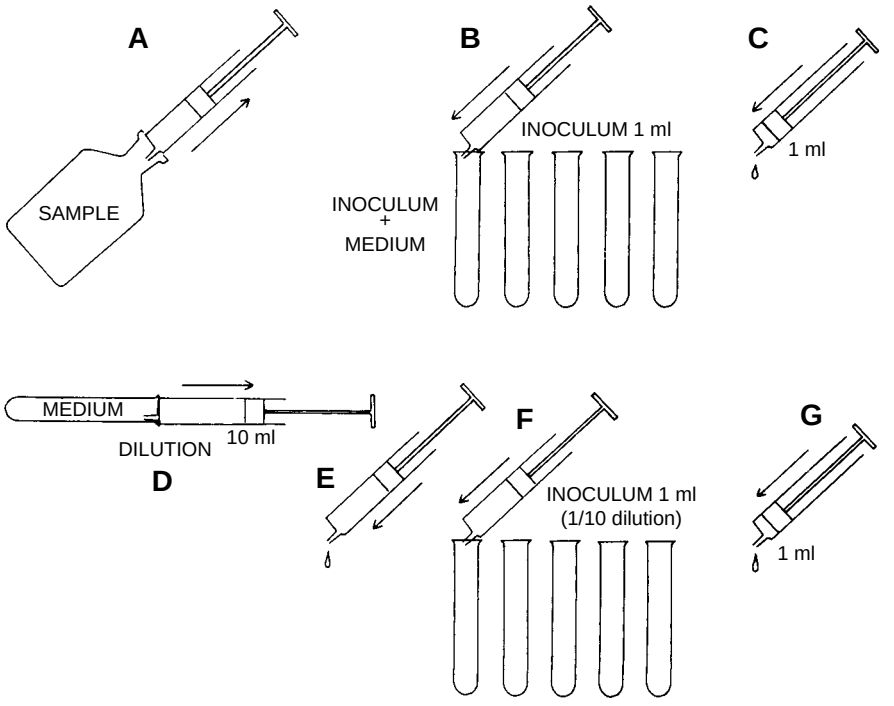

Figure 4.6
Serial dilution culture method, dilutions by syringe. A, subsampling from original water sample; B, inoculation of 1 ml into five test tubes/growth tubes each with 9–10 ml of medium; C, expelling all but 1 ml to prepare for the next dilution; D, making 1/10 dilution by sucking in fresh medium from a test tube filled with medium; E, expelling 1 ml to ensure that the first inoculum is equal to the rest in F, inoculation of 1 ml (diluted sample) into five test tubes/growth tubes each with 9–10 ml of medium; G, expelling all but 1 ml to prepare for the next dilution. Repeat steps D to G for as many dilution steps as required.
Source: adapted from Throndsen (1978).

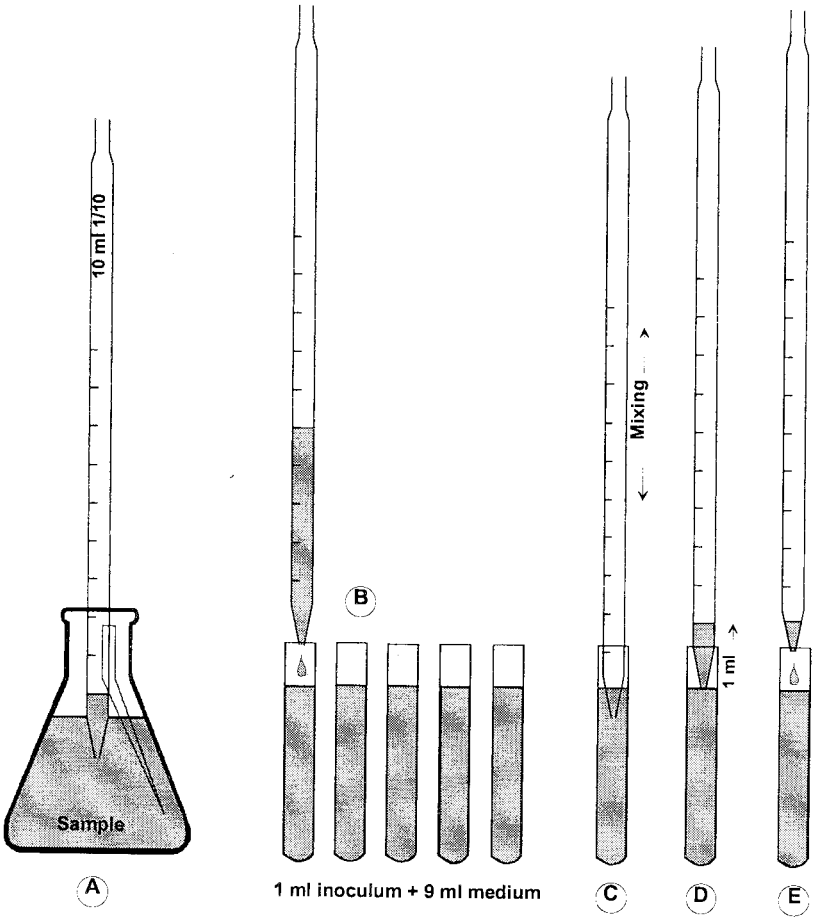

Figure 4.7
Serial dilutions set up with the pipette method. A, subsampling from original water sample; B, adding 1 ml of sample to 9 ml of medium (in five tubes to produce the basic parallels of the series); C, mixing thoroughly; D, withdrawing 1 ml of the suspension into the pipette; E, adding the 1 ml to the next tube with 9 ml medium. Repeat steps C to E to provide for the next dilution steps. (With a new pipette repeat the procedure, starting with the second tube in B, then the third.)

4.12.3 Incubation

The culture series should be placed under fluorescent tubes or daylight at an irradiance of about 10% of full daylight; lower light intensities may be better for deep-sea plankton or benthic species. More detailed information on incubation light intensities can be found in Chapter 3. Take care to keep the temperature variation within the tolerance limits of the phytoplankton species under consideration. Examination (by optical microscope) of the cultures after four and six weeks will often be sufficient for routine work in temperate areas. For microalgae from tropical and subtropical areas, the first examination ought to take place after two weeks, whereas Arctic and Antarctic dilution cultures may need up to two months (at 2–3°C) before cell densities sufficient for further examination under the microscope are established.

4.13 Results

The expected result of a dilution culture series is growth of a variety of the species present in the original sample, with the most abundant species established as unialgal cultures at higher dilutions within the series. Also, in tubes with more than one species present, a particular species may dominate, and by further dilution it can give rise to unialgal cultures. For qualitative and quantitative purposes, the presence or absence of the different species is noted for each of the tubes, starting with the most diluted. These presence and absence data can then be referred to tables for the most probable number (Table 4.9). When estimating cell numbers from Table 4.9, use the set of three successive dilution steps that gives the highest MPN, but make sure that growth has been recorded in at least two of the steps. Note that the SDC method only records cells viable under the culture conditions offered, thus MPN will be minimum values, however, with a standard deviation of ±20–50% of the mean estimate.

Table 4.9 Most probable number (MPN) in original sample. To use the table, compare the presence and absence of the species in question with the values below. Choose the three most diluted steps in which growths occur for the estimate (also see text for advice)

Growth in inoculum			**MPN cells ml^{-1}**		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
5	5	5	>24 000	>2 400	>240
5	5	4	16 000	1 600	160
5	5	3	9 200	920	92
5	5	2	5 400	540	54
5	5	1	3 500	350	35
5	5	0	2 400	240	24
5	4	5	4 300	430	43

…/…

Growth in inoculum			MPN cells ml^{-1}		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
5	4	4	3 500	350	35
5	4	3	2 800	280	28
5	4	2	2 200	220	22
5	4	1	1 700	170	17
5	4	0	1 300	130	13
5	3	5	2 500	250	25
5	3	4	2 100	210	21
5	3	3	1 800	180	18
5	3	2	1 400	140	14
5	3	1	1 100	110	11
5	3	0	790	79	7.9
5	2	5	1 800	180	18
5	2	4	1 500	150	15
5	2	3	1 200	120	12
5	2	2	950	95	9.5
5	2	1	700	70	7
5	2	0	490	49	4.9
5	1	5	1 300	130	13
5	1	4	1 100	110	11
5	1	3	840	84	8.4
5	1	2	640	64	6.4
5	1	1	460	46	4.6
5	1	0	330	33	3.3
5	0	5	950	95	9.5
5	0	4	760	76	7.6
5	0	3	580	58	5.8
5	0	2	430	43	4.3
5	0	1	310	31	3.1
5	0	0	230	23	2.3
4	5	5	810	81	8.1

…/…

Growth in inoculum			MPN cells ml^{-1}		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
4	5	4	720	72	7.2
4	5	3	640	64	6.4
4	5	2	560	56	5.6
4	5	1	480	48	4.8
4	5	0	410	41	4.1
4	4	5	690	69	6.9
4	4	4	620	62	6.2
4	4	3	540	54	5.4
4	4	2	470	47	4.7
4	4	1	400	40	4.0
4	4	0	340	34	3.4
4	3	5	590	59	5.9
4	3	4	520	52	5.2
4	3	3	450	45	4.5
4	3	2	390	39	3.9
4	3	1	330	33	3.3
4	3	0	270	27	2.7
4	2	5	500	50	5.0
4	2	4	440	44	4.4
4	2	3	380	38	3.8
4	2	2	320	32	3.2
4	2	1	260	26	2.6
4	2	0	220	22	2.2
4	1	5	420	42	4.2
4	1	4	360	36	3.6
4	1	3	310	31	3.1
4	1	2	260	26	2.6
4	1	1	210	21	2.1
4	1	0	170	17	1.7
4	0	5	360	36	3.6

…/…

Growth in inoculum			MPN cells ml^{-1}		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
4	0	4	300	30	3.0
4	0	3	250	25	2.5
4	0	2	210	21	2.1
4	0	1	170	17	1.7
4	0	0	130	13	1.3
3	5	5	450	45	4.50
3	5	4	410	41	4.10
3	5	3	370	37	3.70
3	5	2	320	32	3.20
3	5	1	290	29	2.90
3	5	0	250	25	2.50
3	4	5	400	40	4
3	4	4	360	36	3.60
3	4	3	320	32	3.20
3	4	2	280	28	2.80
3	4	1	240	24	2.40
3	4	0	210	21	2.10
3	3	5	350	35	3.50
3	3	4	310	31	3.10
3	3	3	280	28	2.80
3	3	2	240	24	2.40
3	3	1	210	21	2.10
3	3	0	170	17	1.70
3	2	5	310	31	3.10
3	2	4	270	27	2.70
3	2	3	240	24	2.40
3	2	2	200	20	2
3	2	1	170	17	1.70
3	2	0	140	14	1.40
3	1	5	270	27	2.70

…/…

Growth in inoculum			MPN cells ml^{-1}		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
3	1	4	230	23	2.30
3	1	3	200	20	2
3	1	2	170	17	1.70
3	1	1	140	14	1.40
3	1	0	110	11	1.10
3	0	5	230	23	2.30
3	0	4	200	20	2
3	0	3	160	16	1.60
3	0	2	130	13	1.30
3	0	1	110	11	1.10
3	0	0	78	7.80	0.78
2	5	5	320	32	3.20
2	5	4	290	29	2.90
2	5	3	260	26	2.60
2	5	2	230	23	2.30
2	5	1	200	20	2
2	5	0	170	17	1.70
2	4	5	280	28	2.80
2	4	4	250	25	2.50
2	4	3	230	23	2.30
2	4	2	200	20	2
2	4	1	170	17	1.70
2	4	0	150	15	1.50
2	3	5	250	25	2.50
2	3	4	220	22	2.20
2	3	3	200	20	2
2	3	2	170	17	1.70
2	3	1	140	14	1.40
2	3	0	120	12	1.20
2	2	5	220	22	2.20

…/…

Growth in inoculum			MPN cells ml^{-1}		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
2	2	4	190	19	1.90
2	2	3	170	17	1.70
2	2	2	140	14	1.40
2	2	1	120	12	1.20
2	2	0	93	9.3	0.93
2	1	5	190	19	1.90
2	1	4	170	17	1.70
2	1	3	140	14	1.40
2	1	2	120	12	1.20
2	1	1	92	9.20	0.92
2	1	0	68	6.80	0.68
2	0	5	160	16	1.60
2	0	4	140	14	1.40
2	0	3	120	12	1.20
2	0	2	91	9.10	0.91
2	0	1	68	6.80	0.68
2	0	0	45	4.50	0.45
1	5	5	240	24	2.40
1	5	4	220	22	2.20
1	5	3	190	19	1.90
1	5	2	170	17	1.70
1	5	1	150	15	1.50
1	5	0	130	13	1.30
1	4	5	220	22	2.20
1	4	4	190	19	1.90
1	4	3	170	17	1.70
1	4	2	150	15	1.50
1	4	1	130	13	1.30
1	4	0	110	11	1.10
1	3	5	190	19	1.90

…/…

Growth in inoculum			MPN cells ml^{-1}		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
1	3	4	170	17	1.70
1	3	3	150	15	1.50
1	3	2	130	13	1.30
1	3	1	100	10	1
1	3	0	83	8.30	0.83
1	2	5	170	17	1.70
1	2	4	150	15	1.50
1	2	3	120	12	1.20
1	2	2	100	10	1
1	2	1	82	8.20	0.82
1	2	0	61	6.10	0.61
1	1	5	140	14	1.40
1	1	4	120	12	1.20
1	1	3	100	10	1
1	1	2	81	8.10	0.81
1	1	1	61	6.10	0.61
1	1	0	40	4	0.40
1	0	5	120	12	1.20
1	0	4	100	10	1
1	0	3	80	8	0.80
1	0	2	60	6	0.60
1	0	1	40	4	0.40
1	0	0	20	2	0.20
0	5	5	190	19	1.90
0	5	4	170	17	1.70
0	5	3	150	15	1.50
0	5	2	130	13	1.30
0	5	1	110	11	1.10
0	5	0	94	9.40	0.94
0	4	5	170	17	1.70

…/…

Growth in inoculum			MPN cells ml^{-1}		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
0	4	4	150	15	1.50
0	4	3	130	13	1.30
0	4	2	110	11	1.10
0	4	1	94	9.40	0.94
0	4	0	75	7.50	0.75
0	3	5	150	15	1.50
0	3	4	130	13	1.30
0	3	3	110	11	1.10
0	3	2	93	9.30	0.93
0	3	1	74	7.40	0.74
0	3	0	56	5.60	0.56
0	2	5	130	13	1.30
0	2	4	110	11	1.10
0	2	3	92	9.20	0.92
0	2	2	74	7.40	0.74
0	2	1	55	5.50	0.55
0	2	0	37	3.70	0.37
0	1	5	110	11	1.10
0	1	4	91	9.10	0.91
0	1	3	73	7.30	0.73
0	1	2	55	5.50	0.55
0	1	1	36	3.60	0.36
0	1	0	18	1.80	0.18
0	0	5	90	9	0.90
0	0	4	72	7.20	0.72
0	0	3	54	5.40	0.54
0	0	2	36	3.60	0.36
0	0	1	18	1.80	0.18
0	0	0	0	0	0

Source: based on data from American Public Health Association (1955).

4.14 Comments

The procedure may be adopted for different purposes and the set-up can be varied, see above. When cell concentrations are high (>10^6 cells l^{-1}) more dilution steps are added in order to obtain unialgal cultures. If the aim is to clean up or isolate an organism already in culture, the original cell density will determine which dilution steps are critical; dilute so that the inoculum contains one or two cells, use many parallel dilutions, and add one or two further dilution steps. Provided growth occurs only in some of the tubes, the most diluted culture is likely to be clonal, unless cells in the inoculum are clumping.

The quality of the inoculum, the type of medium and the growth conditions offered will determine the success of the method. This selectivity may be used deliberately by choosing a medium which facilitates the growth of particular organisms. For general purposes, a modified Erd-Schreiber medium (Throndsen 1978, 1997) appears to be suitable for coastal waters, whereas a diluted Guillard f medium (Guillard and Ryther, 1962) as (f/2–f/50) may prove better for more oligotrophic areas.

The advantage of the SDC method is that a dominant species may easily be brought into culture provided that it accepts the conditions offered for growth. Not all harmful species fulfil this, for example no *Dinophysis* species has been brought into permanent culture so far. Numerous other taxa, such as *Prymnesium parvum* and several *Chrysochromulina* species, have however been grown through this method. The disadvantage of the SDC method is that less-common species will show up in mixed cultures only. But, drawing upon differences in growth strategies, further SDC series may produce unialgal cultures for the less common species also. As for the MPN estimates, the obvious selectivity and dependence on growth conditions is an important disadvantage, but for many of the species in question the SDC method is the best way to obtain numerical information about cell concentrations and community structure. In the cultures as in nature, not all cells will be typical, but as the quantification is determined by observing the presence or absence of a species, it is not critical that every single specimen in the sample examined be identified. Note, however, that presence or absence of taxa at higher levels has to be deduced from the species (level) in order to obtain a reliable estimate.

Three conditions are particularly important when estimating MPN in mixed cultures:

(a) The level of extinction by dilution may be obscured by competition between different species and/or the effect of algal viruses. Both factors are likely to be inversely related to the dilution.
(b) With growth in all tubes at the highest dilution, the estimate will always be too low and the MPN value should be given a prefix > to indicate that the value is assumed to be less than the real number in the sample surveyed. If possible, this problem could be avoided by adding a sixth dilution step with 1 µl inoculum.
(c) In almost every SDC series there is a serious lack of information on the least-common species encountered in the series.

4.15 Conclusions

At present the SDC is the only simple method of cultivating dominant phytoplankton species, and for the unarmoured nanoplankton taxa it is the easiest way to estimate the number of viable cells. More sophisticated methods such as flow cytometry may

be more precise as regards cell numbers, but demand fairly high operating skills and limited possibilities for species identification. The success or failure of the method depends on (a) the cleanliness of the equipment; (b) the suitability of the growth medium; and (b) the external culture conditions (temperature and light). Experience has shown that in coastal waters the species present are fairly halotolerant, but for some reason SDC prepared with water from the sampling site has proven to have a higher species diversity than those prepared with standard pre-made growth medium.

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5

Detection of HAB species using lectin, antibody and DNA probes

C. Scholin, E. Vrieling, L. Peperzak, L. Rhodes and P. Rublee

An understanding of harmful algal bloom (HAB) phenomena depends greatly on detection and enumeration of specific species found in discrete water samples. Microscope-based cell-identification methods have long been the standard by which this task is accomplished. Although invaluable, traditional microscopy can be time-consuming and requires a certain level of expertise to discriminate key morphological features indicative of HAB species. In some cases, discriminating between 'harmful' and 'benign' organisms is not possible using morphological characters alone. For example, *Alexandrium tamarense* includes both toxic and non-toxic genetically distinct strains distributed throughout the globe (e.g. Scholin, 1998*b*). Identification of toxic and non-toxic representatives of that species can only be accomplished using subcellular criteria.

For many years researchers have sought new means for discriminating among very closely related but genetically distinct species and novel techniques to increase the rate at which specific organisms are quantified, particularly in the context of field surveys. The biotechnology revolution has pointed to the use of molecular probes as being one way to accomplish these goals. As used here, the phrase 'molecular probes' refers to a suite of biological molecules that encompass lectins, antibodies and DNA. Each class of probe shares the ability to selectively adhere to molecules specifically associated with a particular species or group of species, thus serving as a basis for detecting specific organisms even when they occur in complex natural communities (e.g. Scholin and Anderson, 1998).

The first review detailing the use of molecular probes as tools to aid the identification of HAB species was presented by Anderson (1995). Since that time, the use of molecular criteria for characterizing and detecting HAB species has increased dramatically. For some HAB species and in certain areas of the world, the use of lectin, antibody and DNA probes has progressed from relatively limited laboratory trials to routine application for screening numerous cultured and natural samples. In some regions, application of molecular probes is recognized as a valid basis for monitoring as well as conducting HAB risk assessments.

Development of probes for HAB species and methods for their application is an active area of research worldwide. This chapter is not intended to be a detailed review of the many emerging probing technologies and the large number of targeted organisms driving that effort. Instead, our objective is to illustrate how some probes and application methodologies are used currently and to highlight some of the costs and benefits associated with these techniques. Background information concerning probe development, initial testing and preliminary field

verification is found in references cited throughout this chapter. A recent review of molecular detection strategies for HAB species was also recently compiled by Litaker and Tester (2002).

5.1 Application of probes to whole cells and cell homogenates

Probes for HAB species are presently applied using whole-cell or cell-homogenate formats. A general introduction and overview of these concepts is found in Scholin and Anderson (1998) and specific examples of these techniques are presented below. Detection strategies that utilize the whole-cell approach demand that target species remain intact throughout the assay procedure. Samples are typically chemically preserved prior to sample processing. Fluorescently labelled probes selectively bind to specific indicator molecules found on the cell surface or within the confines of the cell wall. Target species of interest are 'labelled' as probes accumulate specifically at sites corresponding to indicator molecules. Fluorescently labelled cells can be revealed by epifluorescence microscopy or flow cytometry. Ancillary information such as cell shape, size, etc., is sometimes used to help to discern a true species-specific reaction from non-specific labelling or interfering autofluorescence. At present, whole-cell assays for HAB species employ lectins, antibodies and ribosomal RNA (rRNA)-targeted DNA probes. A slight variation on the whole-cell labelling technique involves the use of antibodies that recognize specific cell surface markers and facilitate a colorimetric reaction. By measuring the intensity of colour development, the abundance of an organism may be estimated without having to look at individual cells using microscopy or flow cytometry (D. Caron, pers. comm, 2000).

In contrast to whole-cell labelling, cell homogenate-based probe-application techniques require cell lysis. Molecules that serve to indicate the presence of particular species are liberated into solution. Probes are then applied to the crude homogenate or fractions thereof that have undergone some purification to enrich for indicator molecules. By detecting and quantifying these molecular signatures, the presence of the target species can be inferred and in some cases its abundance in the original sample can be estimated. Probes directed against ribosomal rRNA and the genes that encode them (rDNA) are frequently applied in cell homogenate formats.

5.2 Fluorescein (FITC)-conjugated lectin probes

Lectins are non-enzymatic secretory proteins (commonly glycoproteins), derived mainly from a variety of terrestrial sources, which bind non-covalently to specific sugar residues at cell surfaces (Brown and Hunt, 1978; Scholin and Anderson, 1998). Lectins are classified on the basis of their sugar specificity (Slifkin and Doyle, 1990). Potential binding sites associated with microalgae include cell-surface glycoproteins, polysaccharides and chitin (Sengbusch and Müller, 1983; Waite *et al*., 1995; Hori *et al*., 1996).

Application of fluorescently labelled lectins with a range of different binding specificities has been used to differentiate between algal species, even between clones of the same species, for some time (Costas and Rodas, 1994; Costas *et al*., 1993). However, it is only in the last few years that this technique has been seriously

considered as a HAB monitoring and risk-assessment tool. A list of common lectin probes is provided in Table 5.1.

TABLE 5.1 Readily obtainable FITC-conjugated lectins used as probes

Name	Source	Specificity
ConA	*Canavalia ensiformis*	methyl α-D-mannopyranoside; D-mannose; D-glucose
DBA	*Dolichus biflorus* (horse gram)	N-acetyl-D-galactosamine
ECA	*Erythrina cristagalli* (coral tree)	α-lactose; N-acetyl-D-galactosamine; D-galactose
HPA	*Helix pomatia* (snail)	N-acetyl-D-glucosamine; N-acetyl-D-galactosamine; D-galactose
PEA	*Pisum sativum* (pea)	methyl α-D-mannopyranoside; D-mannose; D-glucose
PHA	*Phaseolus limensis* (lima bean)	N-acetyl-D-galactosamine
PNA	*Arachis hypogaea* (peanut)	α-lactose; D-galactose
PWM	*Phytolacca americana* (pokeweed)	N-acetyl-D-glucosamine
RCA	*Ricinus communis*	β-D-galactose
SBA	*Glycine maxima* (soy bean)	N-acetyl-D-galactosamine; D-galactose; methyl α-D-galactopyranoside
UEA	*Ulex europaeus* (gorse)	L-fucose
WGA	*Triticum vulgaris* (wheat germ)	N-triacetylchitotriose; N-diacetylchitobiose; sialic acid

5.2.1 Example protocol and required equipment for applying lectins to whole cells

Fresh solutions of FITC-conjugated lectins (Table 5.1; 100 μg ml^{-1}; Sigma Chemical Co.) are made with filtered synthetic seawater (0.4 μm Nucleopore; Sigma Chemical Co), salinity 28 ppt, pH 8.0. Cell cultures (preserved or live) are filtered following the method of Miller and Scholin (1998) developed for the application of rRNA-targeted probes, and lectins are then added to the filter stack at ≈1 ml per 10^5 cells and incubated for 15 min at 20°C. Unbound lectin is removed by rinsing (synthetic seawater) the sample one or more times, using the vacuum manifold to facilitate exchange of the solutions. Filters bearing treated cells are mounted on glass slides and reactivity assessed by epifluorescence microscopy (excitation, 490 nm; long-pass emission, 520 nm; e.g. Fig. 5.1). The assay is simple to perform, and rapid

(~30 min). The FITC-conjugated lectins vary widely in price per milligram (~US$8 for ConA, ~US$13 for PWM and RCA, and up to ~US$60 for ECA and ~US$72 for HPA; Sigma Chemical Co.). It may be possible to reduce the cost of lectin probes by performing the FITC conjugation in your own laboratory.

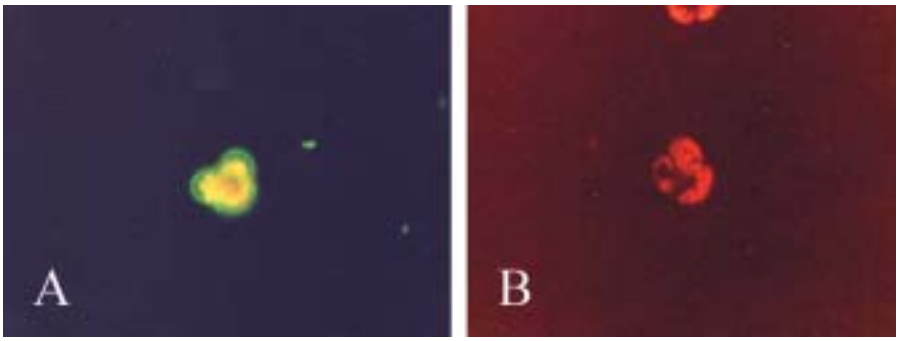

Figure 5.1
Epifluorescence micrograph of *Gymnodinium mikimotoi* isolated from New Zealand (A) with and (B) without addition of FITC-labelled PHA (blue excitation, long-pass filter).

5.2.2 Case studies employing lectin probes

Lectins have been used successfully to discriminate between Spanish strains of toxic *Gymnodinium catenatum* and the morphologically similar, but non-toxic, *G. impudicum* (Costas and Rodas, 1994). In Korea, blooms of the ichthyotoxic *Cochlodinium polykrikoides* have caused massive economic losses to the fish and shellfish industry over the last 20 years (Kim, 1998) and now Red Tide Alerts are issued when cell numbers reach 1,000 cells ml^{-1}. *C. polykrikoides* can be confused under the light microscope with a non-toxic chain-forming *Gymnodinium* species and lectins can be used as a discriminatory tool (Cho *et al*., 1998, 2000). Rhodes *et al*. (1995) and Cho *et al*. (1998) have also used lectins to differentiate between morphologically similar *Karenia* species, and demonstrated that *K. mikimotoi* from New Zealand exhibited a different lectin-binding profile than the same species isolated from Japan and Korea.

The use of lectins for the differentiation of toxic and non-toxic *Pseudo-nitzschia* species has also been explored and is promising for some species in particular geographic regions. Using a suite of lectins, Rhodes (1998) was able to discriminate between six of seven *Pseudo-nitzschia* species; *P. delicatissima* and *P. pseudodelicatissima* differed in binding pattern from the other species, but not from one another. As the latter two species have similar maximum levels of toxicity in the New Zealand strains tested to date (Rhodes *et al*., 1998*a*), the lack of discrimination between them is not considered to be an issue in relation to risk-assessment criteria. Similarly, Fraga *et al*. (1998) discriminated between Galician isolates of seven *Pseudo-nitzschia* species, although binding patterns for the same species isolated from New Zealand and Galician waters differed (Rhodes, 1998). Cho *et al*. (1999) elucidated differential lectin-binding patterns for Korean isolates of four *Pseudo-nitzschia* species, but there were differences between New Zealand, Galician and Korean isolates.

Different lectin-binding profiles were demonstrated for Korean strains of toxic *Alexandrium tamarense* and *A. catenella* and the non-toxic *A. fraterculus* (Cho *et al*., 1999). Lectin-binding profiles of *A. minutum* isolated from Spain (Costas and Rodas, 1994) differ from those of the same species isolated from a New Zealand (Rhodes *et al*., 1995). Like *Pseudo-nitzschia*, reactivity of lectin probes towards *Alexandrium* species varies inter- and intraspecifically, so knowledge of these patterns is essential when using these probes for routine monitoring.

5.2.3 Summary of lectin probes

Binding patterns can differ for 'strains' of the same (morphologically defined) species. In some cases binding profiles may vary depending on the geographic origin of the organisms in question. Some dinoflagellate and cyanobacterial species do not appear to alter lectin-binding profiles as cells progress through different phases of the division cycle, enter exponential or stationary phase, or experience a variety of different environmental conditions (e.g. light, temperature: Alvarez *et al*., 1998; Rhodes *et al*., 1995; Costas *et al*., 1993). However this observation does not hold true for all species of concern. For example, the accumulation of cell-surface sugars in some diatoms does vary depending on growth rate and physiological conditions (Waite *et al*., 1995). Despite some obvious limitations, lectin probes do show promise in particular contexts. The discrimination of *C. polykrikoides* from the morphologically similar *Karenia* species in Korean waters is a good illustration of how lectins can be used for routine monitoring, keeping in mind that such results cannot necessarily be extrapolated to different geographic regions.

5.3 Antibody probes

Antibodies are one of the most common types of probe used in HAB research (Vrieling and Anderson, 1996; Scholin and Anderson, 1998; Peperzak *et al*., 2000). Antibodies bind to molecules collectively referred to as 'antigens'. Antigens include peptides, glycoproteins, carbohydrates, toxins, etc. – molecular constituents of cell walls, membranes, components of cytoplasm, cellular exudates and so forth. Of the antibodies specifically developed for HAB species (Table 5.2), many have been tested in a laboratory setting but only a few have been applied in field studies. At the time of writing, none of the antibodies developed to detect HAB species are available commercially. Consequently, they must be obtained from individual researchers directly involved in the development, testing and application of these tools. It is also possible to prepare novel antibodies *de novo*, but a description of this methodology falls outside the scope of this chapter. Techniques for preparing antibodies can be found in several useful papers and books (e.g. Campbell *et al*., 1989; Harlow and Lane, 1988; Hudson and Hay, 1989).

5.3.1 Polyclonal versus monoclonal antibodies

Serum from an animal inoculated with an antigen contains polyclonal antibodies (PAbs), a suite of antibodies that will bind to many different epitopes. PAbs (or polyclonal antiserum) are sometimes sufficient for detecting the species of interest. However, PAbs may exhibit unwanted cross-reactivity as some antigens may be shared

among different organisms. The specificity of PAbs can be increased by pre-adsorbing them with non-target cells, removing the cross-reacting antibody types (Mendoza *et al*., 1995). In addition to potential problems associated with cross-reactivity, the supply of PAbs can be limited. For example, one rabbit produces about 40–50 ml of crude antiserum and this volume of material may not be sufficient to sustain monitoring programmes for extended periods. Multiple and/or larger animals such as sheep or horses can be used to obtained correspondingly larger volumes of serum, but batch-to-batch PAb preparations do vary in reactivity depending on the individual animal. Ultimately the supply of any given batch of polyclonal serum is finite.

Monoclonal antibodies (MAbs) differ from PAbs in that they are reactive to only a single epitope and are obtained from cultured cells rather than live animals. MAbs are prepared by fusing antibody-producing cells from the host animal with a culturable cell line. Pure cultures of these hybrid cell lines can be maintained indefinitely (in theory) and large batches of specific MAbs with a known reactivity can be produced routinely. MAbs can offer advantages over PAbs when issues of epitope specificity and long-term supply are of concern.

TABLE 5.2 Established antibodies for identifying HAB species; also see references

Species	**Type of antibody**[a]
Bacillariophyceae	
Pseudo-nitzschia pungens	PAb
P. multiseries	PAb, MAb
Cyanophyceae	
Microcystis spp.	PAb
Chrysophyceae	
Aureococcus anophagefferens	PAb
Pelagophyceae	
Aureoumbra lagunensis	PAb
Raphidophyceae	
Chattonella antiqua	MAb
C. marina	MAb
Dinophyceae	
Alexandrium catenella	MAb
A. tamarense	PAb, MAb
A. fundyense	MAb
A. lusitanicum	PAb
A. minutum	PAb
Gymnodinium catenatum	PAb
G. nagaskiense	MAbs[b]
Gyrodinium aureolum	MAb
Prorocentrum lima	PAb
P. minimum	PAb

a. MAb: monoclonal antibody; PAb: polyclonal antibody.
b. Two different monoclonals were established; see Nagasaki *et al.* (1991) and Vrieling *et al.* (1994).
Sources: Vrieling and Anderson (1996); Scholin and Anderson (1998); Peperzak *et al.* (2000).

5.3.2 Primary and secondary antibodies

Antibodies that bind directly to the target molecule (PAb or MAb) are referred to as 'primary'. Primary antibodies can be coupled to a signal molecule, such as a fluorescent dye or enzyme, so that their reaction with an antigen can be tracked (Fig. 5.2A). However, in many cases primary antibodies are not labelled but instead are revealed by the application of a 'secondary antibody', an antibody that reacts specifically with a particular class of primary antibodies (Fig. 5.2B). Secondary antibodies are widely available commercially coupled with a variety of reporter molecules (e.g. fluorescent dyes, enzymes, biotin, gold, etc.). Application of secondary antibodies constitutes an indirect method of labelling. Many immuno-staining techniques developed for identifying HAB species employ the indirect-labelling method using a fluorescent secondary antibody. Another version of the secondary-labelling protocol is known as 'enhanced' (Fig. 5.2C). Enhanced assays are used to increase the number of reporter molecules per antibody, thus elevating the overall signal output (Vrieling *et al*., 1993).

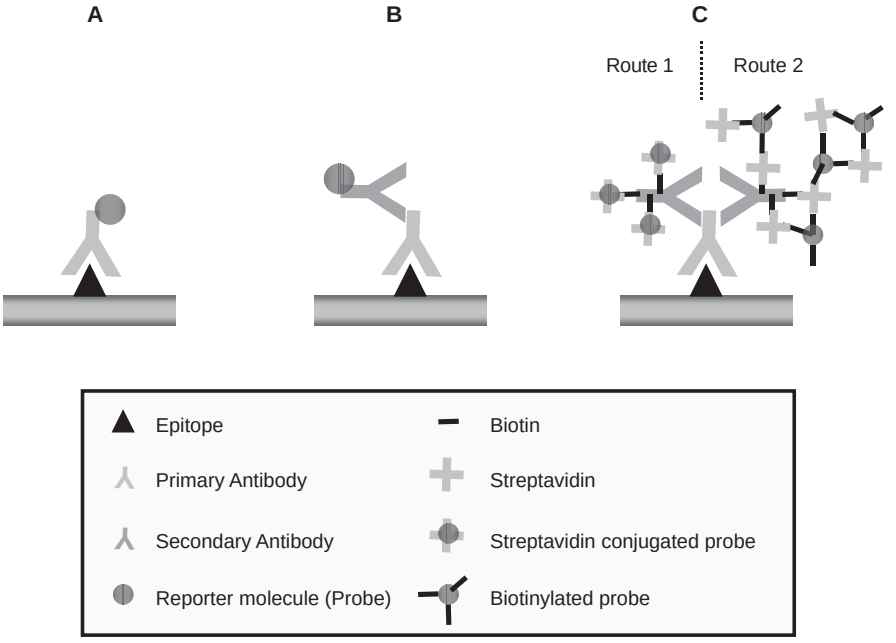

Figure 5.2
Antibody labelling strategies: A, direct; B, indirect; C, enhanced.

5.3.3 Protocols for labelling whole cells with antibodies

Protocols used for immuno-labelling are very similar. Cells are typically preserved chemically, antibodies applied, and products of the reaction viewed by microscopy, flow cytometry, spectrophotometry, etc. The time that antibodies are allowed to react with a sample varies depending on the antibody itself and the assay format (in tubes or on filters) in which it is employed.

All immuno-fluorescence assays start with a blocking step, followed by application of the primary antibody and subsequent washes. The steps that follow will vary depending on the specific type of assay in question: direct, indirect, or enhanced (Fig. 5.2). The direct assay is rapid, requiring just a single incubation with a labelled antibody followed by one or more rinses. The indirect assay is about twice as long, due to incubation with a secondary antibody and an additional series of washes. Enhanced assays are the most time consuming and require the most sample manipulation.

General descriptions of indirect antibody-labelling methods are given below. Tube assays use centrifugation as a means of concentrating cells between reagent exchanges. Filter assays are very similar, except that they employ a filtration vacuum manifold to concentrate cells and facilitate reagent exchange. The protocols given begin with a preserved sample. Examples of the type of results obtained using *Gymnodinium mikimotoi* as the target species are shown in Fig. 5.3.

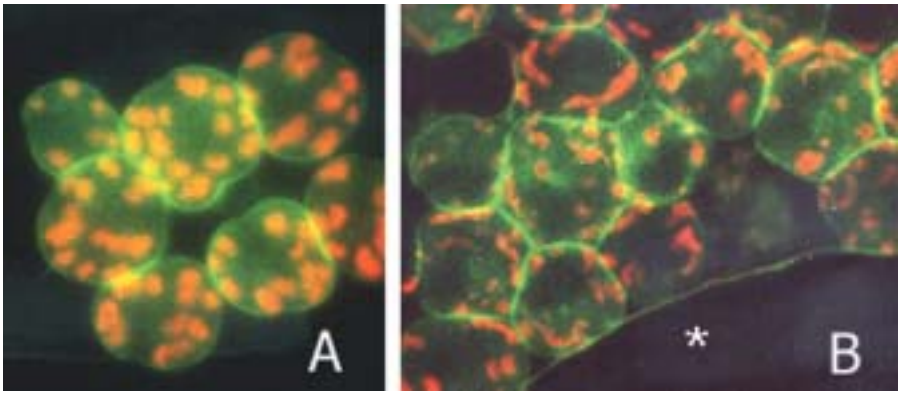

Figure 5.3
Karenia mikimotoi labelled with an FITC-conjugated primary MAb using (A) 'tube' and (B) 'filter' processing formats (blue excitation, long-pass filter). Chloroplasts are visible as the yellow/red bean shapes within the cell walls. The asterisk indicates a bubble trapped under the cover slip.

5.3.3.1. *Tube assays*

- Concentrate cells by centrifugation; rinse twice with phosphate-buffered saline (PBS).
- Block non-specific binding sites by adding 2.0 ml PBS containing 1.0% (w/v) bovine serum albumin (BSA); incubate for 5 min at room temperature.
- Centrifuge, remove supernatant (leaving a small volume on top of the cells) and re-suspend cells in PBS containing the diluted primary antibody; incubate 1 h at room temperature.
- Centrifuge, rinse at least twice with PBS and re-suspend cells in PBS containing the diluted secondary probe-conjugated antibody (see recommendations of manufacturer); incubate for 1 hour at room temperature.
- Centrifuge, rinse at least twice with PBS and re-suspend cells in PBS for analysis (0.1–1.0 ml and 0.5–5.0 ml for microscopy and flow cytometry, respectively).

Remarks:

1. The duration and centrifugal force applied during the centrifugation steps can be altered to suit the size and stability of the target species.
2. The concentration of the primary antibody must be determined empirically.
3. Dilute samples can be pre-concentrated (by slow-speed centrifugation, sieving or sedimentation) to allow the use of smaller sample processing tubes; smaller reaction volumes (<2 ml) increase the speed of the assay.
4. The extent of cell loss varies with cell size (Vrieling *et al*., 1996); swing-out rotors help to diminish loss (Vrieling *et al*., 1995*a*).
5. Tube assays are needed if preparing samples for flow cytometry.

5.3.3.2 *Filter assays*

- Load manifold filtration apparatus with polycarbonate filters, concentrate cells by applying a vacuum and rinse with 5.0 ml PBS to remove excess fixative; avoid letting the filter go dry between reagent exchanges.
- Add 5.0 ml PBS containing 0.5% (w/v) BSA; let stand for 10 min at room temperature. A little back-pressure at the outlet will prevent gravity flow of fluid; filtration stacks can also be fitted with stopcocks to control fluid flow (e.g. Miller and Scholin, 1998).
- Wash the sample twice with 5.0 ml PBS, add PBS containing the diluted primary antibody and incubate for 30 min at room temperature.
- Remove excess primary antibody by washing twice with 5.0 ml PBS, add PBS containing the diluted secondary probe-conjugated antibody (see recommendations of manufacturer) and incubate for 30 min at room temperature.
- Remove excess secondary antibody by washing twice with 5.0 ml PBS (the filter should become nearly dry after the final filtration step), and disassemble the filtration unit.
- Mount filters, sample side up, on a slide using one drop of mounting medium (glycerol, immersion oil, or antifade reagent) in the middle of the filter, then examine using epifluorescence microscopy.

Remarks:

1. Filtration manifolds are available commercially (e.g. Millipore) or can be assembled on a custom basis (Miller and Scholin, 1998; Peperzak *et al*., 1998*b*).
2. Filters are typically 13 mm or 25 mm in diameter.
3. The best results are obtained using polycarbonate filters or alternatively those made from cellulose nitrate. Black filters are useful when autofluorescence of standard filters interferes with sample analysis.
4. Occasionally filters may fall dry accidentally. Generally this is not a serious problem, but some deformation of cell morphology may be observed. The amount of mounting medium is generally 10 µl, but this may need to be adjusted.
5. Samples can be stored by sealing the edges of the cover slip with nail polish, or by freezing the slides at -20°C. With the latter method, FITC-labelled *K. mikimotoi* cells retained signal even after six months of storage.

5.3.3.3 *Enhanced immuno-labelling assays*

The enhanced immuno-labelling method (Fig. 5.2C) can be used in conjunction with tube and filter-based sample-processing formats. The procedure starts as above,

except that the secondary antibody is labelled with biotin instead of a fluorescent molecule. The remaining steps of the enhanced assay technique are given below:

- Remove excess secondary antibody by washing twice with 5.0 ml PBS, then add PBS containing either diluted probe-conjugated streptavidin (route 1 Fig. 5.2C; Vrieling *et al*., 1993) or streptavidin and biotin-conjugated probe (route 2 Fig. 5.2C); dilutions should follow manufacturer's recommendations.
- Incubate for 30 min at room temperature, wash twice with 5.0 ml PBS and analyse the samples.

Remarks:

1. Schemes 1 and 2 as shown in Fig. 5.2C are highly simplified, as multiple-probe molecules are conjugated to streptavidin (route 1) and biotin (route 2).
2. In route 2 (Fig. 5.2C) the complexation of streptavidin and biotinylated probe may result in non-specific aggregation. Therefore, additional controls (using only the secondary antibody and the complexation constituents) are necessary to ensure that the reaction observed is specific.

5.3.4 Equipment and considerations for detecting immuno-labelled cells

An epifluorescence microscope must be equipped with appropriate excitation and emission filters, depending on the particular fluorescent dye attached to the probe. Familiarity should be sought with the autofluorescent characteristics of organisms routinely seen in samples. Heterotrophic dinoflagellates, for example, may appear green using a long-pass emission filter (e.g. LP520, standard for FITC; Shapiro *et al*., 1989) and this signal may be confused with a fluorescein-labelled probe. It is also possible to identify multiple species simultaneously using different probes each labelled with a unique fluor. Again, potential interference resulting from autofluorescence must be borne in mind. For standard photography, sensitive films (ASA $\geq$400) are preferred to minimize exposure times to avoid photo bleaching of the sample. Digital cameras offer one of the easiest means of photo documentation, and digital images are highly amenable to qualitative/quantitative analysis using any one of a number of commercial software packages. Overall, results obtained using microscopy depend a great deal on the quality of the microscope, light source, objectives and filter sets. A high-quality research-grade epifluorescence microscope can cost US$25,000 or more, not necessarily including photo-documentation equipment.

Alternative methods for detecting immuno-labelled cells include flow cytometry and fluorescence as well as standard spectrophotometry. Flow cytometers vary widely in price and capability, but can easily exceed US$100,000. Flow cytometers can provide physical data of individual cells using forward- and side-scattering detectors; multiple fluorescence detectors are possible as well (Peperzak *et al*., 2000). Flow cytometry also offers the capacity for statistical analysis of the spectral properties of labelled populations (e.g. fluorescence intensity, size, shape) even when they occur in mixed populations (Vrieling *et al*., 1996). Standard and fluorescence spectrophotometers also vary widely in capability and price, with many units falling in the US$10,000–25,000 range when an ability to read a microwell plate is included. Fluorescence spectrophotometry is at present not used for routine detection of immuno-labelled cells. Ultimately this method may help to speed analysis by processing samples using 96 well plates (e.g. Vrieling *et al*., 1994). The volume of

samples that can be processed in 96 well plates is limited, however, so this method is not necessarily applicable to detecting HAB species at low concentrations in field samples.

5.3.5 Immunoassay fixatives

Formaldehyde (formalin): purchased as 37% (w/v) stock solution and diluted to a final concentration between 0.2% and 2%. These solutions may contain a fraction of methanol which may affect cell membrane integrity and autofluorescence.

Paraformaldehyde: prepared in stocks from 10–40% (w/v) by depolymerizing solid paraformaldehyde under alkaline conditions. Used at final concentration between 0.2% and 2%. The stock solution deteriorates quickly; do not use stocks older than one month.

Glutaraldehyde (GA): purchased as stock solutions of 25% or 50% (v/v) and maintained at 4°C for extended periods. Used at final concentration between 0.2% and 2%. GA seriously affects autofluorescent characteristics of phytoplankton cells, inducing yellowish/green fluorescence; chlorophyll autofluorescence diminishes over time.

Iodine solutions: two types, of which one is acidic (Lugol's version) and the other alkaline (Utermöhl's version) used in concentrations of 1.0–4.0‰ (v/v). Delayed analysis of iodine-preserved sample may lead to loss of algal cells and autofluorescence. The acidic solution dissolves calcified structures, such as coccoliths, which may contain antibody-targeted molecules.

Alcohols: ice-cold methanol or ethanol are used at concentrations from 65% to 96% (v/v). Ethanol is sometimes combined with sodium chloride to increase its ionic strength. Alcohol will permeabilize membranes. This is an advantage for labelling intracellular antigens or when performing *in situ* hybridization with DNA probes (see Section 5.4), but may also lead to cell loss during sample processing. Alcohol treatments can drastically diminish pigment autofluorescence.

Freezing: liquid nitrogen is used effectively, but cell damage may occur. Freezing may be more useful when combined with prior chemical fixation (e.g. Vaulot *et al*., 1989; Vrieling *et al*., 1994).

5.3.6 Immunoassay blocking agents

Blocking agents mask non-targeted sites that might otherwise adsorb primary or secondary antibodies and lead to unwanted reactions. Typical formulations of these solutions include bovine serum albumin (BSA), normal serum and milk powder.

BSA: albumin is a protein that is present in serum of all animals and does not interact with antibodies (except antibodies raised against serum albumin itself!). Working concentrations vary from 0.5% to 2.0% (w/v). BSA is available in many degrees of purity, and for most applications less highly purified forms are suitable; highly purified grades are preferred in conjunction with electron microscopy applications.

Normal serum: serum from the same animal species from which the primary and/or secondary antibodies were obtained. Normal serum contains many proteins (including albumin) and is obtained from foetal stages not influenced by novel immune responses. If the amount of pre-immune serum (i.e. that from an animal immediately prior to immunization) is limited, normal serum can be used in its place.

Milk powder: condensed milk powder (available in most supermarkets) works very well in immuno-assays where blotting or spectrophotometric methods are applied. Concentrations of 0.1–2.0% (w/v) can be applied without interference. The use of milk powder is not recommended in conjunction with microscopy and flow cytometry, because it can be difficult to wash away excess quantities; increased washing steps needed to remove this material will lead to increased cell loss during sample processing.

5.3.7 Common immunoassay buffers

Buffers used for antibody incubations and washing must not interfere with antibody/antigen interaction or affect the integrity and morphology of targeted cells. Two common buffers that meet these requirements are phosphate and TRIS-buffered saline. Sodium azide (NaN_3) can be added to buffers and antibody stocks at a concentration of 0.01–0.05% (w/v) to reduce bacterial or fungal growth. Sodium azide is typically prepared and stored as a concentrated stock solution (e.g. 1000×), then diluted to working strength as needed.

Phosphate-buffered saline (PBS): dissolve 0.23 g $NaH_2PO_4 \cdot H_2O$, 1.44 g $Na_2HPO_4 \cdot 2H_2O$, 8.0 g NaCl, and 0.2 g KCl per litre of distilled water; pH 7–8. In some applications the detergent Tween-20 (~0.05% v/v) or Triton X100 (~0.1%v/v) is used and the buffer is then referred to as PBST. Glycine (10 mM) can be added and the pH set between 7 and 8; this formulation is referred to as PBSG.

TRIS-buffered saline (TBS): dissolve 1.2 g of TRIS base and 1.75 g NaCl in 900 ml distilled water. Adjust pH between 7 and 8 with concentrated HCl and then add water to 1 l. The concentration of TRIS may vary between 10 mM (1.2 g l^{-1}) to 50 mM (6.0 g l^{-1}). Tween-20 may be added (as for PBS), in which case the formulation is referred to as TBST.

5.3.8 Cell-permeabilizing agents used for immuno-labelling

For some applications it may be necessary for antibodies to penetrate cells in order to label specific molecules found within the confines of the cell wall or organelles (Lin *et al*., 1995; Lin and Carpenter, 1996). Detergents such as saponin and Triton X–100 (both at concentrations < 1%) are often useful for this purpose, even when applied to formaldehyde-fixed cells. Saline ethanol (Miller and Scholin, 2000; see Section 5.4.2) or methanol also enhances the permeability of cells.

5.3.9 Case study: antibody probes for detecting *Karenia mikimotoi*

A number of harmful *Alexandrium* spp., *Dinophysis* spp., raphidophytes and *Karenia mikimotoi* (previously referred to as *Gyrodinium aureolum*) as well as nuisance species (*Phaeocystis globosa*, *Noctiluca scintillans*) often co-occur in Netherlands coastal waters (Vrieling *et al*., 1995*b*). A rapid method that utilized flow cytometry to detect specific target species was desired. To meet that need for *K. mikimotoi*, Vrieling *et al*. (1994, 1995*a*) developed and tested a series of MAbs using cultured and natural samples. Species identification based on immunolabelling was in good agreement with identifications based on microscopy. However, deterioration of cells in Lugol's fixative and loss of cells during sample processing using the indirect-labelling/tube-assay technique hampered accurate quantification of *K. mikimotoi*. By conjugating the primary antibody with FITC directly, thus

eliminating sample handling associated with a secondary antibody, cell loss was minimized. This direct-labelling technique is now used to identify *K. mikimotoi* at densities of about 1,000 cells/l in a tube-assay format combined with flow cytometry (Vrieling *et al*., 1996, 1997) and < 100 cells l^{-1} in a filter assay followed by epifluorescence microscopy (Peperzak *et al*., 1998). Although the two methods differ in sensitivity, *K. mikimotoi* can readily be detected at concentrations below those of concern. These assays have been used for routine detection of *K. mikimotoi* in Netherlands coastal waters since 1996.

5.3.10 Considerations when using antibodies

It is critical to adjust the antibody concentration to a level that assures all target cells are labelled maximally. Development of antibody assays for *K. mikimotoi* (Vrieling *et al*., 1997) and *A. tamarense* (Anderson *et al*., 1999) are examples of how antibody concentrations are 'tuned'. Cross-reactivity studies are also essential to establish the specificity of antibodies. Culture collections are extremely useful in this regard and it is advisable to evaluate the antibodies against as many strains of the target species as possible (including those from different geographic locations), as well as related species and organisms that may commonly co-occur with the target species in nature. Compared with wild cells, laboratory cultures may display altered cell-wall molecules. For example, MAbs used to detect *Chattonella* spp. label cultured cells brighter than when the same species is collected from natural populations; wild cells appear to be encased in a layer of mucilage which masks antigens on the cell surface and limits reactivity with the antibody (Nagasaki, pers. comm; Vrieling *et al*., 1995*a*). The choice of sample preservative will affect antibody/antigen reactions as well (Vrieling and Anderson, 1996). Lugol's iodine or osmiumtetraoxide often dramatically decreases labelling intensities. Aldehyde fixatives (especially [para]formaldehyde) are better at maintaining labelling intensity, although glutaraldehyde often introduces an interfering autofluorescence which cannot always be discriminated from an overlapping reporter signal. Fluorescent dyes are available with a wide range of excitation and emission spectra, making it possible to select dyes that minimize interfering autofluorescence (Haugland, 1999).

Fluorescein (FITC) is one of the most common dyes used for immuno-labelling of HAB species. Heterotrophic dinoflagellates will exhibit green autofluorescence when viewed with a filter-set optimized for FITC. Strong chlorophyll autofluorescence can also obscure FITC-specific signals. Extraction of chlorophyll or removal of cell contents can considerably boost the apparent signal. Simultaneous application of multiple antibody preparations directed at the same species (Vrieling *et al*., 1994), or use of antibodies in the enhanced-signalling format (Vrieling *et al*., 1993), are alternative means of strengthening the signal. Labelling of the cell wall of *Prorocentrum micans*, for example, is much brighter when PAbs directed against the cell wall as well as those against trichocysts are applied simultaneously.

Prolonged exposure to a high-powered excitation light source can lead to photo bleaching, or the loss of fluorescence from the reporter molecule. Bleaching is most problematic when samples are analysed using microscopy, since loss of fluorescence hampers timely examination and enumeration of labelled cells. Bleaching effects can be retarded using a number of antifading agents, such as citifluor, DABCO, n-propyllgalate and Slowfade Light (Vrieling and Anderson, 1996; Haugland, 1999).

Rates of sample processing depend a great deal on the type of assay being performed (e.g. tube versus filter) and the labelling strategy employed. For example, using a filter-based format up to 15 samples can be processed in about 45 min, 90 min, or 135 min for the direct, indirect and enhanced assays, respectively. Tube assays require more time due to the handling of the tubes during centrifugation steps. The time required to evaluate the filters using microscopy will depend on cell density, the number of target cells and the amount of interfering matter (e.g. detritus, particulate matter, autofluorescence), but an experienced person may on average spend about 10 min examining a particular filter. It is possible to process as many as 30–50 samples per day. Sometimes it is useful to process samples in batches, performing the labelling reactions on one day and examining the results of those reactions in the next few days. Use of a flow cytometer speeds up analysis of samples processed in batch mode, with interpretation of those data following thereafter.

5.3.11 Immuno-labelling summary

A number of reports describe the use of immuno-chemical methods for detecting HAB species in field samples (e.g. Uchida *et al*., 1989; Adachi *et al*., 1993; Anderson *et al*., 1993; Vrieling *et al*., 1995*a*, 1995*b*; Blasco *et al*., 1996; Peperzak *et al*., 2000). Despite its promise, the approach of using antibodies as tools for identifying a wide range of species appears to be under-utilized. This may be due to the lack of commercial availability of suitable, species-specific antibodies, as well as a misconception that antibody-labelling techniques are technically difficult to perform. Intraspecific variation in reactivity towards an antibody preparation is also possible. Nevertheless, once an antibody assay is established for a given species as it occurs in a given geographic location, its application can become routine and cost-effective, around US$5–10 per sample in expendable supplies. Charges for labour and overheads will increase the total per sample analytical costs.

5.4 Fluorescently labelled, rRNA-targeted DNA probes for identifying whole cells

Oligonucleotide (DNA) probes for identifying HAB species applied in the whole-cell format have been directed against sequences of small subunit (18S or SSU), large subunit (28S or LSU) and the intergenic transcribed spacers (ITS1, ITS2) of the rRNA cistron (e.g. Adachi *et al*., 1996; Anderson *et al*., 1999; Miller and Scholin, 1996, 1998; Rublee *et al*., 1999). Much of this work, especially as it relates to field surveys, has focused on species of *Alexandrium*, *Pseudo-nitzschia* and *Pfiesteria* (e.g. Parsons *et al*., 1999; Rhodes *et al*., 1998*a*, 1998*b*; Scholin *et al*., 1997; Miller and Scholin, 1998; Rublee *et al*., 1999; Scholin *et al*., 2000; Anderson *et al*., unpublished). In New Zealand, this technique is formally integrated in a nationwide programme of monitoring for toxic species of *Alexandrium* and *Pseudo-nitzschia*; this is the first-ever government-sanctioned use of probes for conducting HAB risk assessments.

Detailed examples of how one develops and tests fluorescently labelled rRNA-targeted DNA probes may be found elsewhere (see for example Adachi *et al*., 1996; Anderson *et al*., 1999; Miller and Scholin 1996, 1998, 2000; Rublee *et al*., 1999; Litaker and Tester, 2002). Design of conventional oligonucleotide probes rests on

obtaining and comparing rRNA and/or rDNA sequences from the organisms of interest (target and non-target species). Sequences of ~20± bases that are potentially unique to the target species are identified and synthetic oligonucleotide probes directed against those sequences are synthesized, typically using a commercial vendor specializing in oligonucleotide production. Similar to antibodies, oligonucleotide probes can be labelled with a variety of fluorescent dyes, the choice of which may vary depending on the spectral characteristics of the target species or interfering materials (debris, non-target species). Examples of DNA probes used to label whole cells are given in Table 5.3; none of these probes are commercially available at the time of writing.

5.4.1 Protocols for labelling whole cells with rRNA-targeted DNA probes

Methods for identifying HAB species using fluorescently labelled DNA probes are very similar to methods that use lectins and antibodies. A sample is collected and preserved chemically. Ideally, the fixative chosen will preserve the morphology of the target species, reduce interfering autofluorescence, stabilize nucleic acids and permeabilize the cell membrane to allow oligonucleotide probes access to rRNA contained within the confines of the cell wall. Typical fixatives include formaldehyde or paraformaldehyde followed by methanol treatment (e.g. Anderson *et al*., 1999; Rublee *et al*., 1999), or a saline ethanol solution (Miller and Scholin, 2000). In cases where the target species is rare, samples may be pre-concentrated by filtration or sieving prior to preservation. Visualization of the target species is accomplished using epifluorescence microscopy. Use of control probes provides a reference for judging the reactivity of the species-specific probe. For example, a positive control probe binds to rRNA found in all organisms whereas a negative control probe does not (Table 5.3, Fig. 5.4; see Miller and Scholin, 1996, 1998, 2000). A 'no probe' control is useful for assessing the autofluorescent characteristics of a sample, and when compared with the negative probe-control treatment the extent to which probes may be binding non-specifically can be judged.

Like methods for applying lectins or antibodies, labelling of intact cells using oligonucleotide DNA probes requires several exchanges of reagents during the fixation, labelling and washing steps of the procedure. This can be accomplished using centrifugation to concentrate cells (Adachi *et al*., 1996; Scholin *et al*., 1996; Anderson *et al*., 1999; Rublee *et al*., 1999), or gentle vacuum filtration using commercial or custom-built filter manifolds. The home-built filtration system of Miller and Scholin (1998) can also be used to collect and store samples prior to processing. For many diatom and dinoflagellate species, preserved samples can be stored in the fixative or even be dried and stored for many weeks prior to labelling (Miller and Scholin, 2000).

An example of a filter-based, DNA probe whole-cell labelling protocol is given below. Alternative protocols for conducting whole-cell staining with rRNA-targeted probes may be found elsewhere (Adachi *et al*., 1996; Anderson *et al*., 1999; Rublee *et al*., 1999). The method detailed here works well for labelling species of *Alexandrium* and *Pseudo-nitzschia*, but can be used to label many other species as well. The procedure begins by assuming that the sample was preserved using either the aldehyde/methanol or saline ethanol treatments and is ready for processing. Prior to this, the sample would have been collected and fixed in the filter tube itself, or collected and preserved in a separate procedure and then added to the filter stack immediately

before labelling (see for example Miller and Scholin, 2000). The filter tubes are fitted with 13 mm polycarbonate membranes (Millipore Isopore, Whatman Cyclopore, etc.) whose pore size may range from 1–5 µm. Filter stacks can also be fitted to accept 25 mm filters, in which case the volumes of reagents used must be adjusted slightly higher so that the sample is completely flooded at each step.

- Collect sample on to the filter using the least vacuum possible, then rinse once using 1–2 ml 5× SET hybridization buffer. Allow sample to stand in hybridization buffer at room temperature for several minutes.
- Vacuum-concentrate sample, then add ~500 µl hybridization buffer to each tube to re-suspend cells before adding 12 µl of appropriate probe (probe stock at 200 ng μl^{-1}). Alternatively, probes can be pre-mixed in hybridization buffer at a final concentration of ~5 ng μl^{-1} and added directly to the sample as a single component. Incubate samples at the appropriate temperature in a dry incubator or water bath for 15 min to 2 hours.
- Concentrate sample as before and add 1–2 ml 5× SET pre-warmed to hybridization temperaure to remove excess unbound probe. Incubate for several minutes; apply vacuum to withdraw rinse solution.

TABLE 5.3 Examples of DNA probes used for whole-cell hybridization analysis of cultured and natural samples

Probe[a]	**Sequence (5'–3')**[b]	**Hybridization temperature**[c] **(°C)**	**Species targeted**[d]
uniC	GWATTACCGCGGCKGCTG	45	positive control
uniR	CAGCMGCCGCGGUAAUWC	45	negative control
NA1	AGTGCAACACTCCCACCA	45	*Alexandrium tamarense* (North American ribotype)[e]
auD1	AAATGACTCACTCCACCAGG	45	*Pseudo-nitzschia australis*
puD1	ATGACTCACTTTACCA	45	*P. pungens*
muD1	ATGACTCACTCTGCCA	45	*P. multiseries*
muD2	AAGCCCACAGCGCCCAAGCC	55	*P. multiseries; P. pseudodelicatissima*
heD2–2	TATCCACAGCGCCCACA	45	*P. heimii*
frD1	AAAGACTCATTCTACCAGG	45	*P. fraudulenta*
deD1	AGACTCACTCTACCA	45	*P. delicatissima*
amD1	ATGACTCATTCAGCCA	45	*Nitzschia americana*

a. uniC: positive control, SSU-targeted universally conserved sequence (519r; Field *et al.*, 1988; Embley *et al*., 1992); uniR is the complement of uniC. Base position ambiguities: W = T and A; K = T and G; M = C and A. NA1 has also been used as a negative control when probing for *Pseudo-nitzschia* spp. (see Scholin *et al*., 1996).
b. Complement of targeted rRNA sequence, except for uniR.
c. Using a 5× SET hybridization buffer and application protocol as outlined in Section 5.4.1.
d. Reference strains from Monterey Bay, Calif., USA.
e. See Scholin, 1998a.
Sources: Miller and Scholin (1996, 1998, 2000).

- Place filter, sample side up, on microscope slide, add 20 µL SlowFade Light (Molecular Probes, Eugene, Ore.) and mount cover glass.
- View samples using an epifluorescence microscope fitted with an appropriate filter set (see Fig. 5.4). Mounted filters can be stored in the refrigerator for several days (e.g. Miller and Scholin, 2000).

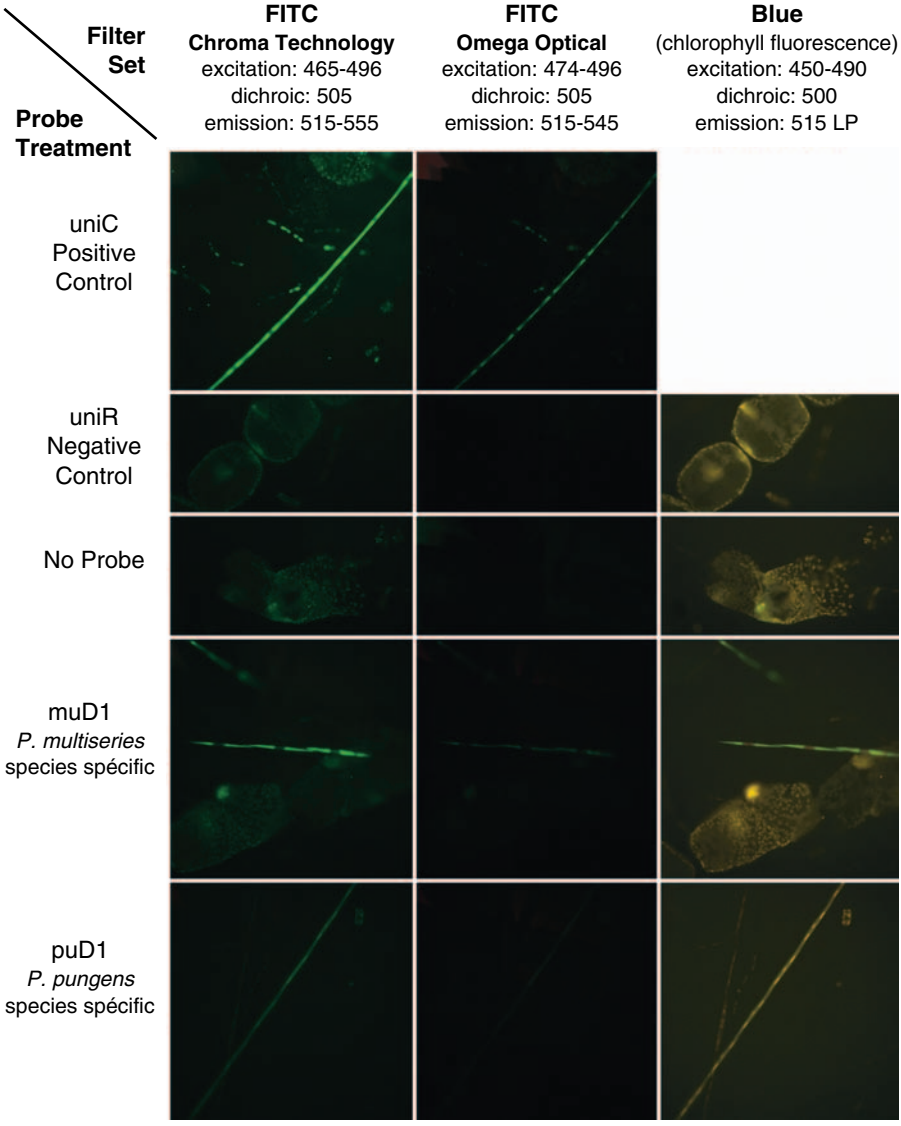

Figure 5.4
Examples of epifluorescence micrographs of cells stained with FITC-labelled, rRNA-targeted probes (Table 5.3). Note how the quality of the stains and interfering autofluorescence changes depending on the particular filter set used. Photos by P.E. Miller.

Remarks:

1. The appropriate hybridization temperature will vary depending on the ionic strength of the hybridization buffer, the sequence of the probe itself and the extent to which the targeted species varies genetically from non-target organisms in the same sample.
2. Hybridization reactions should be kept as short as possible; long incubation times sometimes result in increased background.
3. For some probes it may be necessary to rinse with solutions of lower ionic strength, such as 0.2–1.0× SET, to achieve the desired specificity of a given probe.
4. Use of multiple probes, each labelled with a unique fluor, can be applied simultaneously to detect multiple species in a single sample (see Miller and Scholin, 2000).

5.4.2 Common reagents used for labelling whole cells with fluorescent DNA probes

The solutions listed below are used in conjunction with the protocol given in Section 5.4.1. When mixing solutions, use standard sterile technique and RNase-free glassware, plasticware and reagents.

25× SET (3.75 M NaCl, 25 mM EDTA, 0.5 M TRIS pH 7.8): to make 250 ml add 54.8 g NaCl to a 1 l graduated cylinder, add 125 ml 1 M TRIS pH 7.8, add 12.5 ml 0.5 M EDTA, bring volume to 250 ml using dH_2O. To make the NaCl dissolve, use a stir plate and stir bar. Sterile filter using a Nalgene 0.2 µm filter unit. Store at room temperature.

Saline ethanol fixative: for 30 ml of standard fixative combine 25 ml high-grade, native 95% ethanol (should not contain methanol), 2 ml distilled H_2O, 3 ml 25× SET. For 30 ml of modified fixative combine 22 ml high-grade, native 95% ethanol (should not contain methanol), 5 ml distilled H_2O, 3 ml 25× SET. Note: samples stored in standard fixative for longer than a few hours may develop precipitates. The 'modified fixative' is stable for months at room temperature (see Miller and Scholin, 2000). Alteration of ethanol and salt concentrations may improve fixation of some species.

5× SET hybridization buffer: for 240 ml hybridization buffer use a 250 ml Nalgene 0.2 µm filter unit and combine 189.0 ml distilled H_2O, 48.0 ml 25× SET, 2.4 ml 10% IGEPAL-CA630 (Sigma Chemical Co.). After sterile filtering add 0.6 ml polyA (Sigma Chemical Co.; nucleic acid, 12.5 mg ml^{-1}). Stocks of polyA are prepared by re-suspending dried polyA in distilled, RNAse-free water. The concentrated polyA stock can be aliquoted in sterile, RNase-free micro centrifuge tubes and frozen at -20°C until needed.

Fluorescent probes: probes ordered from a commercial vendor are shipped dry. Care should be taken to protect probe stocks from light. A concentrated stock can be prepared by re-suspending dried material at 1 µg $µl^{1}$ in distilled, RNAse-free water. The concentrated stocks can be frozen for short-term storage. For long-term storage, aliquot concentrated probe stock to micro centrifuge tube and dry in a vacuum centrifuge (e.g. SpeedVac); store dried probes dessicated at -70°C protected from light. Working stocks of probes can be prepared by re-suspending dried probes or diluting concentrated probe stocks in TE buffer (10 mM TRIS, 1 mM EDTA, pH 8.0) at 200 ng $µl^{-1}$. Probes may be pre-mixed in hybridization buffer to a final concentration of 5 ng $µl^{-1}$; store refrigerated, protected from light.

5.4.3 Considerations when labelling whole cells with DNA probes

Many of the same problems associated with the application of lectin and antibody probes for identifying species also apply to rRNA-targeted probes. The perceived quality of the stain can vary a great deal depending on the microscope used, its objectives, filter set, light source, etc. (Fig. 5.4). Adapting labelling protocols for analysis of field samples often involves scaling of sample volumes to minimize interference from background material. For example, 10–25 ml of whole water, or several hundred microlitres of net tow material, are generally 'optimal' sample volumes when attempting to enumerate *Pseudo-nitzschia* species using filter-based whole-cell hybridization. Larger sample volumes can result in accumulation of material (cells and debris) that may obscure target species. An abundance of detritus or non-target species can also interfere with detection of a targeted species by forcing a reduction in sample volume, thus reducing assay sensitivity (fewer labelled cells). When the target species is rare (e.g. 100 cells l^{-1} or less), then the sample may have to be pre-concentrated prior to processing and/or analysis of replicate samples may be required to quantify that organism effectively. Labelling intensity may also vary, possibly due to fluctuations in cellular rRNA content or permeability of the cell wall towards the probe. Genetically divergent strains of the same (morphologically defined) species may not react equivalently towards a 'species-specific' probe. It is critical that probes for a given species be matched to geographic regions where those probes are applied.

The cost of applying rRNA-targeted probes for staining whole cells can vary from about US$1–5 per sample in expendable supplies depending on the number of probes used, the number of replicate samples analysed, etc. The probes are the least expensive component of the assay, costing around a mere US$0.10 per application. Filters and expendable plastics make up the bulk of the cost of expendable supplies. The time required to view a filter and count specific species varies depending on the nature of the sample and the experience of the person examining it. A single person might process 10–20 samples per day, but this can be gruelling work if that pace is sustained for long periods. Total sample-processing time is approximately 2–3 hours, with about an hour being hands-on. Labour fees can significantly increase costs if those charges are considered part of the total per sample analytical expenditures. An epifluorescence microscope or flow cytometer are required.

The DNA-based whole-cell labelling approach is not necessarily universally applicable to identifying all HAB species. Detection of some raphidophytes, particularly *Heterosigma akashiwo* and *Fibrocapsa japonica*, illustrates some of these problems. Adequate fixation of the cells, both to preserve morphology and promote probe staining, has proven problematic (Tyrrell *et al*., 2001). Clumping of labelled cells can also interfere with their enumeration.

5.5 Sandwich-hybridization application of ribosomal RNA-targeted DNA probes

The sandwich-hybridization technique involves collection of a sample onto a filter, followed by addition of a chaotropic solution and heat to break cells and liberate nucleic acids (Van Ness and Chen, 1991; Van Ness *et al*., 1991). The resulting sample homogenate (unpurified cell lysate) is then dispensed to a pre-packaged test plate

and processed automatically in a relatively simple benchtop system (Scholin *et al*., 1997, 1999). Two separate hybridization reactions ensue: capture of target nucleic-acid sequences (DNA or RNA) from the crude lysate using an oligonucleotide tethered to a solid support, and binding of a signal probe to a sequence near that of the capture site. Visualization of capture probe/rRNA/signal probe 'sandwiches' is accomplished enzymatically, yielding colorimetric or chemiluminescent products. The extent of colour development or chemiluminescence can provide a measure of the abundance of target species in the original sample (Scholin *et al*., 1999, 2000). Total sample-processing time, from live sample concentrated on a filter to assay completion, is about 1 hour, of which some 15 min are hands-on sample manipulations. The sandwich-hybridization technique is highly amenable to automation, making autonomous, *in situ* detection of target species possible (Scholin *et al*., 1998). As with other cell homogenate-based detection schemes, sandwich hybridization offers a potentially faster mode of sample processing than whole-cell-based assays, especially when large numbers of samples must be processed rapidly. Detection of multiple species in a single sample simultaneously is also possible (e.g. Scholin *et al*., 1999).

Sandwich-hybridization assays have been devised for *Alexandrium tamarense/catenella/fundyense* (North American, Western European and temperate Asian strains), *Pseudo-nitzschia australis*, *P. multiseries*, *P. pseudodelicatissima*, *P. pungens* (reference strains from central California), *Heterosigma akashiwo*, *Fibrocapsa japonica*, *Chatonella antiqua/subsalsa*, and a Cryptoperdiniopsoid species (from Florida) not yet formally described. At the time of writing, prototype sandwich-hybridization kits are available from the Saigene Corporation (Seattle, Wash.), but the kits are still undergoing an active phase of development, testing and refinement. These products could be readily available by 2003. Cost of expendable reagents used in the assay currently averages around US$5 per sample, per target organism. Specialized equipment required to use the sandwich-hybridization test kits includes a custom robotic processor (can be rented from Saigene for a small fee) and 96 well plate reader (price can vary widely, from US$2,000–25,000). A more simplified 'dipstick' test format (e.g. Scholin, 1998*a*) is being developed. The latter will greatly simplify sample processing and eliminate the need for a 96 well plate reader.

Field testing of sandwich-hybridzation assays has focused primarily on species of *Pseudo-nitzschia* (Rhodes *et al*., 2001; Scholin *et al*., 1999, 2000), North American strains of *Alexandrium tamarense/catenella/fundyense* (Scholin and Anderson, unpublished data), *Heterosigma akashiwo* and *Fibrocapsa japonica* (Rhodes *et al*., 2001, Tyrrell *et al*., 2001). The assay has been used in the laboratory as well as aboard ship. The latter option has provided a means for near real-time mapping of *Pseudo-nitzschia* and *Alexandrium* species.

5.6 PCR application of ribosomal RNA-targeted DNA probes

Similar to the sandwich-hybridization technique, detection methods for HAB species that employ the polymerase chain reaction (PCR) are based on the binding of complementary strands of nucleic acids. In its simplest sense, PCR is multiple nucleic-acid replication events in a test tube rather than within a cell. The process targets only a fragment of the genome, however, based on the use of oligonucleotide

primers that define the size of the fragment as well as the taxonomic specificity of the reaction. The principles and methods governing this reaction are described extensively elsewhere (e.g. Innis *et al.*, 1995; Ausubel *et al*., 1995). The assays require PCR primers, extraction of nucleic acids from sample material, and application of one or more amplification protocols. Here, direct, real-time and heteroduplex mobility assay PCR detection methods (Fig. 5.5) are discussed using *Pfiesteria* as an example target organism.

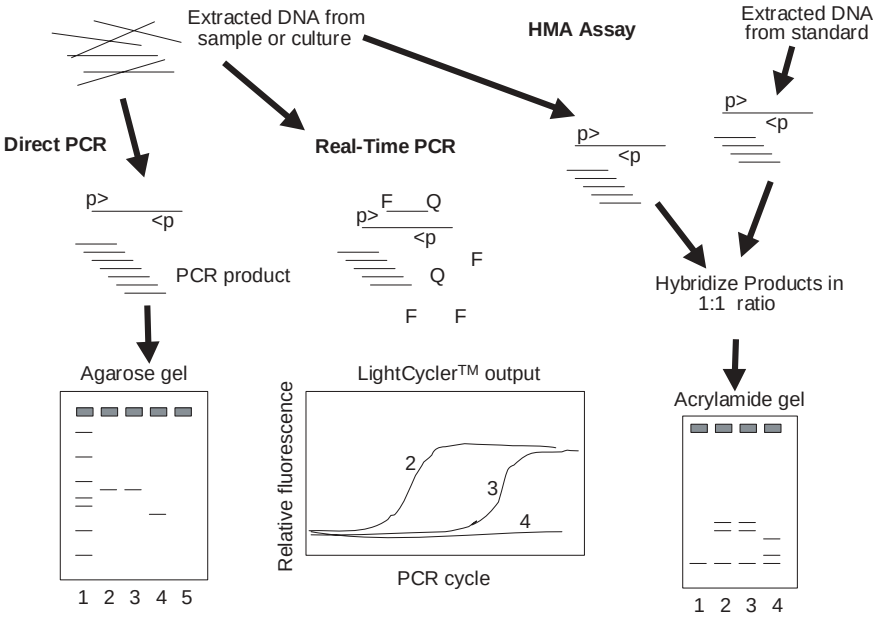

Figure 5.5
Schematic of (left) direct polymerase chain reaction (PCR), (centre) real-time PCR and (right) heteroduplex mobility assay (HMA). PCR primers are indicated as p>, <p, and F and Q represent fluor and quencher molecules in the HMA. For the agarose gel output from the direct PCR assay, lane 1 is a size standard and lane 5 is the negative control (no template). In the acrylamide gel output from the HMA assay, lane 1 is a homoduplex derived from hybridizing products from two PCR reactions of the standard culture. In the output from all methods, lane 2 represents the result with a positive control sample, lane 3 represents a typical positive result from a sample and lane 4 represents a negative result from a sample.

The first step in development of any PCR-based technique is determination of an appropriate nucleic acid or gene target sequence that has the desired specificity, whether at species, genus or other taxonomic level. A variety of gene sequences can be used, but ribosomal RNA (rDNA) genes, especially the 18S or SSU rDNA, have been most common as they include highly conserved regions common to most organisms. This is particularly advantageous as primers are available that should be

able to amplify any 18S gene, allowing subsequent sequencing to locate unique sequences within variable regions which can then be targeted as unique molecular 'signatures'. Other targets within the rDNA cistron (ITS and NTS regions) allow specificity at even finer levels (strain-specific primers are possible; see for example Goggin, 1994) because they are not structural genes and therefore are less conserved evolutionarily. A potential disadvantage of using rDNA sequences, especially the SSU rDNA, is that they may not discriminate among different forms of a target species, such as toxic versus non-toxic, or particular life-cycle stages that may have different functional roles in the environment. In this regard, the use of functional gene targets (such as enzyme-encoding genes), and reverse transcriptase (rt) PCR directed toward messenger RNA (mRNA) may yield information more relevant to resolving questions about an organism's toxicity, activity, life stage, etc. (e.g. Pichard and Paul, 1993).

Once a potential signature sequence is identified, a pair of oligonucleotide primers (forward and reverse) are designed to bind to unique sequences within or bordering that target. Successful amplification depends on well-designed primers that have been checked against databases to assure that they are unique and will not cross-react. Further, they must bind effectively to the target, should have similar annealing temperatures (T_m), and should not bind to each other. Software to aid in primer design is available on the Internet or from commercial vendors. Ultimately, empirical testing of primers against cultured material is essential to assure their efficacy and that they do not cross-react to non-target organisms.

5.6.1 DNA extraction

Prior to PCR amplification, DNA must be extracted and purified from the sample, removing potential inhibitors of that reaction and the amplification steps that follow. For culture material or water samples, a volume is generally concentrated by filtration or centrifugation, followed by the extraction using any one of a variety of published methods or commercial extraction kits (e.g. QIAquick™ PCR Purification Kit, QIAGEN Valencia, Calif.; Puregene™ DNA Isolation Kit, Gentra Systems, Minneapolis, Minn.). An example of a method for PCR-based detection of *Pfiesteria* species that uses a CTAB (cetyltrimethylammonium bromide) buffer is given below.

- 20–40 ml of cultured cells or approximately 50 ml of estuarine water are drawn on to a glass-fibre filter by vacuum filtration.
- The filter is immersed in 2 ml of 2× CTAB buffer and may be stored at room temperature for up to six months before extraction. Do not refrigerate the CTAB solution as components will precipitate.
- DNA extraction begins by macerating the filter in the buffer solution (using a sterile wooden applicator); heat to 65°C for 1 h, with occasional mixing.
- Add an equal volume (2 ml) of aqueous chloroform/isoamyl alcohol solution to the sample and mix thoroughly. Centrifuge for about 20 min or until good separation of the aqueous and organic layers is clear; transfer the upper aqueous layer to a clean tube.
- Add 0.7 times the volume of 100% 2-propanol and invert the tube several times. Centrifuge for about 25 min at top speed in a microcentrifuge (~16,000–20,000 × g), remove supernatant and allow DNA pellet to air dry.
- Re-suspend dried residue in 25 µl TE buffer (pH 7.4) and store at -20°C until use.

As dinoflagellates may form cysts and amoeboid stages, sampling of sediment may also be desirable. Sediment sampling is particularly problematic as it is well known that inhibitors in sediments may be carried over in the DNA extract and purification steps, and small amounts of inhibitors are sufficient to poison PCR reactions. For detection of *Pfiesteria* in sediments, DNA from about 0.25 cm^3 of material is typically extracted using a commercial kit (MoBio Ultra Clean™ Soil DNA Kit, MoBio Laboratories, Solana Beach, Calif.).

The efficiency of extraction of all samples is important for PCR, especially if it is to be used in a quantitative or semi-quantitative fashion. Efficiency is based on two processes, the ability of the buffers to lyse cells, and the ability to purify (i.e. remove any inhibitors) and recover target DNA prior to the PCR reaction. Cell lysis may vary among life stages and can only be estimated by spiking the sample with internal standards consisting of intact whole cells at life stages appropriate to what is sought in natural samples. As the chemical character of every sample may be different, ideally each sample should be run both with and without such an internal standard. This approach also resolves the concern about efficiency of purification. An alternative approach to this second concern would be to add an internal control of DNA to the PCR reaction itself, either as an added spike of target DNA in a replicate sample, or in the form of competitive PCR targets (Piatak *et al*., 1993).

Remarks:

1. For DNA extraction it is critical that the type of filter be matched to the chemical constituents of the extraction protocol, because DNA may bind to the filters under some conditions (e.g. chaotropic buffers result in binding of DNA to glass-fibre filters, but not to nylon filters). For commercial kits that use guanidinium-based buffers, use of 5 µm pore size hydrophilic nylon filters is recommended (e.g. Durapore filter, Millipore Corp., Bedford, Mass.).

Commonly used reagents in DNA extractions

CTAB lysis buffer: Dissolve 12.114 g TRIS base in 800 ml double-distilled water and adjust pH to 8.0 with approximately 80 ml 1 N HCl. Add 40 ml 0.5M EDTA solution, 81.8 g NaCl, and 20 g CTAB. Stir while heating in order to dissolve. Do not autoclave.

Chloroform/isoamyl alcohol solution: mix 24 parts chloroform to 1 part isoamyl alcohol. Store in a bottle with 10 ml double-distilled water added which will form a layer on top of the chloroform/isoproponal mixture and keep it hydrated.

TE buffer (pH 7.4): Add 0.5 ml 2 M TRIS-HCl (pH 7.4) and 0.2 ml 0.5 M EDTA (pH 8.0) to 99.3 ml distilled water. Sterilize by autoclaving and store at room temperature.

5.6.2 Direct non-quantitative PCR detection of target DNA

The simplest and most common type of PCR reaction is designed to amplify a target sequence so that it can be visualized on an agarose gel after electrophoresis and staining of the DNA (e.g. Fig. 5.5). Reactions are generally conducted in a thermalcycler (about US$4,000–8,000) and require relatively expensive reagents (US$1–2 per sample) that must be stored frozen at -20°C. A typical PCR reaction mixture contains the purified DNA sample (template), the two primers designed to amplify a target sequence (e.g. Table 5.4), Taq polymerase, mg++ and a mixture of dNTPs, all in a buffer solution. Additional reagents (e.g. BSA, formamide) are sometimes added to enhance the reaction. The reagents in the reaction mixture can be purchased

separately, but most suppliers also offer premixed solutions to which only the template and primers need to be added. A typical PCR reaction protocol for *Pfiesteria piscicida* follows:

- In a 0.5 ml PCR reaction tube, mix 5 µl of 10× PCR buffer (500 mM KCl, 100 mM TRIS-HCl pH 9.0 at 25°C, 1% Triton X–100); 5 µl of 25 mM $MgCl_2$; 1.5 U Taq DNA polymerase; 2.5 µl of 4 mM dNTP stock solution (1 mM each of dATP, dGTP, dCTP, dTTP;); 5 µl of 100 µM bovine serum albumin; and 1 µl of 10 µM solutions of each forward and reverse primer (final concentration of 0.2 µM per primer).
- Add approximately 100 ng of template DNA per reaction.
- Add sterile distilled water to bring the total volume to 50 µl .
- Place the reaction tubes in a thermal cycler and run under the following protocol: denature at 94°C for 2 min; cycle 39 times through 94°C for 1 min, 60°C for 1.5 min, 72°C for 2–3 min, cool and hold at 4°C until analysis by electrophoresis.

Reaction products are generally visualized by agarose gel electrophoresis (followed by staining of the gel with ethidium bromide, SYBR green, or some other nucleic-acid stain (Ausubel *et al*., 1995). The concentration of agarose may be adjusted to match the size of the reaction product of interest. When loading the gel, marking dyes are added to ensure that the product is not lost from the gel before visualization.

Remarks:

1. Thermalcyclers may use tubes or plates, and reaction volumes can be adjusted proportionally, however PCR reaction conditions should be tested empirically in order to optimize the reaction for the desired specificity. Altering the concentration of Mg^{++}, the pH of the reaction mixture, or the annealing temperature, may all have an effect on the quality and quantity of the PCR product.

TABLE 5.4 DNA primer pairs used for PCR-based detection of *Pfiesteria* and *Pfiesteria*-like species

Pfiesteria piscicida primer pairs	
Ppisc–108F	5'-AGTTAGATTGTCTTTGGTGGTCAA-3'
Ppisc-311R[a]	5'-GATAGGTCAGAAAGTGATATGGTA-3'
Ppisc-65F	5'-AGCCTAAGCTTGTTAAACGGCAATGC-3'
18S R[a]	5'-TGATCCTTCTGCAGGTTCACCTAC-3'
Ppisc–110F	5'-GTTAGATTGTCTTTGGTGGTCAATCC-3'
18S R[a]	5'-TGATCCTTCTGCAGGTTCACCTAC-3'
Pfiesteria shumwayae primer pair	
Pshum-F	5'-TGCATGTCTCAGTTTAAGTCCCA-3'
Palt R[a]	5'-TCGAAAGCTGATAGGTCAGAATC-3'
Cryptoperidiniopsis (tentatively *C. brodyi*) primer pair	
CryptF	5'-CTCATTAAAACAGTTCTCGAATA-3'
CryptR[a]	5'-ATAGGTCAGAACCAAAAATGATC-3'

a. rRNA complement.
Sources: Rublee *et al.* (1999); Oldach *et al.* (2000); Bowers *et al*. (2000).

2. Concentration of the target DNA and ratio of target to non-target DNA may be critical to successful PCR. Often the suspended DNA in TE buffer from the extraction process is too concentrated, and samples may need to be diluted 1:10, 1:100 or even 1:1000 to yield robust reaction products.
3. It is critical to run both positive control reactions (a known positive standard) and negative control reactions (all reagents except the template) to assure that reagents have not degraded or been contaminated. Examples of specific reaction conditions and components of the reaction mixture can be found in Bolch *et al.* (1999) and Penna and Magnani (1998, 1999) for *Alexandrium* spp., and in Rublee *et al.* (1999) and Oldach *et al.* (2000) for *Pfiesteria* spp.
4. Annealing temperatures used in PCR cycles must be matched to the primer pair. The melting temperature, T_m, is generally provided by the manufacturer of the primer and the annealing temperature is usually a few degrees lower than the T_m.

5.6.3 Direct quantitative PCR with fluorescent probes

Recently, Bowers *et al.* (2000) described real-time PCR assays for *Pfiesteria* spp. In this assay, an oligonucleotide probe with both a fluorophore and a quencher molecule (Taqman™) are used in addition to oligonucleotide primers (Table 5.5), and an instrument capable of excitation and detection of fluorescent signals is required. Initially in the PCR reaction, when the probe molecule is intact, the quencher prevents fluorescence. However, as the probe (which is complementary to a region in the amplicon) is incorporated into amplicons at each cycle, the exonuclease activity of the Taq polymerase releases the fluorescent and quencher molecules into solution, where the fluorescent molecule can now emit light when excited by light of the appropriate wavelength. Thus, the relative fluorescence is related to the number of amplicons (free fluorescent molecules in solution) and the cycle at which this

TABLE 5.5 Primer/probe combinations for real-time Taqman™ assays of *Pfiesteria* and *Pfiesteria*-like dinoflagellates

Pfiesteria piscicida	
Primer '107'	5'-CAGTTAGATTGTCTTTGGTGGTCAA-3'
Primer '320'	5'-TACCATATCACTTTCTGACCTATCA-3'
P. pisc probe	5'-FAM-CATGCACCAAAGCCCGACTTCTCG-TAMRA-3'
Pfiesteria shumwayae	
Pshum F	5'-TGCATGTCTCAGTTTAAGTCA-3'
Pshum R	5'-TCGATCATCAAATACACTAAAACTGTTTT-3'
P. shum probe	5'-FAM-TACGGCGAAACTGCGAATGGCTCAT-TAMRA-3'
Gyrodinium galatheanum	
G. gal F	5'-CGCAACCCTTGTTTGGTCAG-3'
G. gal R	5'-AGTAAGCGGCTCTTTGTCTTAACC-3'
G. gal probe	5'-FAM-TGAGAAATCGGAGGAAGGTAAGGATGACG-TAMRA-3'

Sources: Bowers *et al.* (2000); Tengs *et al.* (2001).

fluorescence can be detected is directly related to the number of target molecules in the initial reaction mixture (e.g. Fig. 5.5). A related approach uses Molecular Beacons™ (Tyagi and Kramer, 1996), which are also oligonucleotides with fluorophores and quenchers located in close proximity to inhibit fluorescence. In this approach, the molecular beacon hybridizes to single-stranded amplicons in the reaction mixture, which separates the quencher from the fluorophore, leading to quantitative fluorescence relative to the number of target molecules in a fashion similar to that of the Taqman™ probes. Advantages of the real-time quantitative PCR approach are that it uses small samples (7–25 µl total reaction volume), the complete reaction generally takes less than 1 h, the reaction can be monitored in real time (no gels are needed), and sensitivity of detection is increased due to use of the fluorophores. Disadvantages include a more expensive instrument than direct PCR reactions (US$35,000–65,000 or higher), the use of proprietary reaction tubes (with some instruments), and the use of Taqman™ probes or molecular beacons that are more expensive (~ US$200 per probe) than the oligonucleotide primers used in direct PCR.

Briefly, the protocol for real-time quantitative PCR (after Bowers *et al*., 2000) using a LightCycler™ System (Roche Molecular, Mannheim, Germany) is:

- Prepare a reaction mixture (10 µl) containing final concentrations of 0.2 µM of each primer, 0.15 µM fluorescent probe, 0.1 U μl^{1} Taq polymerase, 4 mM $MgCl_2$, 0.2 mM each dNTP, 0.25 mg ml^{-1} BSA, and a 1× final concentration of the PCR buffer supplied with the Taq.
- Add approximately 10 ng template DNA.
- Add sterile distilled water to a final volume of 10 µl.
- Load 7 µl into reaction cuvettes.
- Place the reaction cuvettes in the LightCycler™ and run under the following protocol: 50 cycles at 94°C for 0 s and 60°C for 20 s, with a temperature transition time of 20°C/s. Fluorescence acquisition was 100 m/s after each incubation at 60°C and display mode was CH1/1 with gain set at 1.

Remarks:

1. Not all assays will show the same degree of fluorescence for positive samples. Empirical testing of reaction conditions is essential to optimize assays.

5.6.4 Heteroduplex mobility assays

The heteroduplex mobility assay (HMA) is an alternative PCR-based method that has been used to confirm the validity of species designations associated with cultures and as a tool to discover new strains or species (e.g. Uribe *et al*., 1999; Oldach *et al*., 2000). In this assay two PCR reactions are initially performed using primers that are taxon-specific (genus level or higher) rather than species-specific. One reaction uses a defined template or ‘driver’, while the other consists of a DNA template from an unknown culture or environmental sample. The reaction products are then mixed in a 1:1 ratio, denatured at high temperature and hybridized as the mixture cools. The resultant hybridized amplicons are then electrophoresed through a polyacrylamide gel. If the driver and unknown are identical, all hybrids will be homoduplex DNA amplicons (perfectly complementary strands from the same organism) and a single band will appear on the gel. If the unknown is different from the driver, then both homoduplex and heteroduplex DNA amplicons (nearly complementary strands from two organisms, but containing mismatches) will generate bands on the gel, as

heteroduplexes migrate slower than homoduplexes (e.g. Fig. 5.5). The pattern of the bands will be characteristic of the species or strain, as long as the same driver and reaction conditions are maintained. Uribe *et al*. (1999) utilized primers from the D1–D2 region of the LSU of *Prorocentrum micans* to amplify and compare cultures of *Alexandrium* spp. Oldach *et al*. (2000) utilized a taxon-specific SSU rDNA forward primer selective for dinoflagellates (5'-CGATTGAGTGATCCGGTGAATAA-3') paired with a SSU rDNA universal eukaryotic reverse primer (see Table 5.4) and *Akasiwo sanguinea* as the driver species to aid in determination of the identity and 'purity' of cultures of *Pfiesteria*-like organisms. Additionally, they were able to assess some environmental samples for the presence of *Pfiesteria*. HMA assays are particularly valuable for identifying unknown cultures, determining if they are composed of a single or more species in the taxon of interest, and they can help to direct sequence discovery. Their use in evaluation of field samples may be problematic, however, as a field sample containing multiple species of the taxon of interest will generate multiple bands and can rapidly become impossible to analyse (the number of heteroduplex bands increases geometrically as the number of species increases). The procedure for HMA assays (Oldach *et al*., 2000) follows:

- Duplicate PCR reactions are run, one with a template from a known dinoflagellate 'driver' standard, and one from an unknown culture or field sample. The PCR reaction mixture (50 µl) contains 10–20 ng template DNA, 50 mM KCl, 20 mM TRIS-HCl (pH 8.4), 4.0 mM $MgCl_2$, 200 µM nucleotides, 1 U Taq DNA polymerase, (all in PCR Supermix™, Gibco/Life Technologies, Gaithersburg, Md.) and 0.8 uM of each primer.
- PCR conditions using dinoflagellate-specific PCR primers: 95°C × 3 min; then 40 cycles (95°C for 30 s/55°C for 30 s/72°C for 40 s); then 72°C for 5 min, followed by storage at 4°C until analysis.
- Mix equivalent amounts of reaction products from target and template DNA (100 ng). Denature at 95°C for 5 min, cool rapidly to 68°C and maintain for 30 min, then slowly cool (2°C per min) to room temperature.
- Triple Dye loading buffer (FMC Bioproducts, Rockland, Me.) is added to each tube and the samples are separated by electrophoresis across a 1× MDE gel (FMC Bioproducts) containing 4 M Urea, in a 20 cm Protein II xi electrophoresis unit (BioRad Labs, Hercules, Calif.) at 140 V over 24–36 hours. Gels are stained with SYBR Green II (FMC Bioproducts) for 30 min, and visualized on an Alpha Imager (Alpha Innotech, San Leandro, Calif.) utilizing a SYBR filter.

5.6.5 PCR protocol summary

The review of PCR methods given here is brief and has been directed at three applications. Innovation is still the rule in application of PCR and new approaches that increase sensitivity and flexibility of the assay are constantly being developed. Overall, PCR approaches are proving to be sensitive means of detecting HAB species in environmental samples, as a means of confirming identity and purity of cultures, and as a means of directing sequence discovery. They may be particularly useful with cryptic species, species where strain differences may be a critical component of hypothesis resolution, and for species such as *Pfiesteria* which may constitute only a small proportion of total dinoflagellates in the water column even when it is causing a problem (Burkholder and Glasgow, 1997). The methods require considerable 'up-front' effort to assure sensitivity and specificity of the assays, and their application

may be problematic in some environmental samples, especially if the chemical nature of the sample inhibits DNA extraction and purification. They require at least a modest investment in equipment (thermalcycler, gel electrophoresis equipment, real-time PCR machines for advanced applications), with costs that might range upwards of US$25,000. Reagents used for PCR can be expensive too. Once PCR-based assays are established, however, they can provide rapid and extremely sensitive analyses.

5.7 Conclusions

Molecular-based methods for detecting HAB species are used routinely in many laboratories around the world. No single type of molecular probe or assay strategy appears to stand out as being 'optimal'. Indeed, some HAB species can be detected using a variety of probes. The choice of probe for a given species in a given region seems to follow individuals' personal preference, technical background and available laboratory equipment.

It is critical that probes be 'tuned' to suit the geographic region of interest, regardless of the type of probe or application format. Many species exhibit diversity on a molecular scale that is not always apparent morphologically; probes that work well to identify a particular organism in one geographic region may not work well to identify the same (morphologically defined) organism in another. Thus, reactivity of probes towards target and non-target species must be established empirically, region by region, and molecular characterization of those species should be carried out in conjunction with morphological-based analyses. Given this broad perspective, it is apparent that 'species-specific' probes may not exist in the strict sense of that phrase. For example, there is no single *Alexandrium tamarense*-specific probe because the species exists as a series of molecularly distinct strains recognizable through application of lectin, antibody and rRNA-targeted probes. Thus, descriptions of the reactivity of a probe should include both species and strain designations whenever possible. In that regard, establishing an internationally accepted standard for naming 'molecular strains' of morphologically defined species would be beneficial.

With the exception of lectins and the prototype sandwich-hybridization assays, none of the probes discussed above are available commercially. The lack of readily available probe 'kits', complete with control samples, limits the extent to which these tools are applied outside those laboratories directly involved with probe development and testing. This situation is likely to change with the increased demand and use of the probes for routine monitoring.

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6

Cyst methodologies

D. M. Anderson, Y. Fukuyo and K. Matsuoka

Many marine phytoplankton species produce dormant cysts or resting spores during their life histories. Alternation between a dormant, benthic stage and a motile, vegetative existence is a complex process that must be considered in our efforts to understand and manage blooms of harmful algal species. Cyst germination provides an inoculum for many blooms, and cyst formation can subsequently remove substantial numbers of cells in later stages. Such cells have other important ecological roles with respect to species dispersal, survival through adverse conditions and genetic recombination when sexuality is involved in their formation (Wall, 1971). Among the toxic or harmful marine phytoplankton species are many that use this life history strategy. Some are listed in Table 6.1, although it should be recognized that many unlisted

TABLE 6.1 Some toxic or harmful species that produce resting cysts

Marine species	**Reference**
Dinophyceae	
Cochlodinium sp.	Fukuyo (1982)
Cochlodinium sp.	Matsuoka (1985, 1987)
Gymnodinium catenatum	Anderson *et al.* (1988); Matsuoka (1987)
Alexandrium catenella	Yoshimatsu (1981)
A. cohorticula	Fukuyo *et al.* (1990)
A. minutum	Bolch *et al*. (1991)
A. monilatum	Walker and Steidinger (1979)
A. ostenfeldii	Bolch *et al*. (1991)
A. tamarense (= *A. excavatum*)	Dale (1977); Anderson and Wall (1978)
Pyrodinium bahamense var. *compressum*	Steidinger *et al*. (1980); Matsuoka *et al*. (1989b)
Raphidophyceae	
Chattonella antiqua	Imai and Itoh (1988)
C. marina	Imai and Itoh (1988)
Heterosigma akashiwo	Imai and Itakura (1991)

(non-toxic) species that also produce cysts can cause harm due to dense biomass accumulation and anoxia. Here we present a compilation of methodologies useful in laboratory and field studies of phytoplankton resting cysts and spores. Methods for dinoflagellate cysts dominate the presentation, as most cyst-forming species belong to this group and it has therefore been studied most thoroughly. Information relevant to other algal classes are also presented where appropriate.

6.1 General concepts, definitions

6.1.1 Cysts

Most toxic or harmful species reproduce by asexual, binary division. Under certain conditions, however, sexuality is induced, involving a series of developmental events that produce morphologically and physiologically distinct cell types called gametes, zygotes and hypnozygotes (reviewed in Pfiester and Anderson, 1987). The term 'cyst' is used to describe a non-motile cell that lacks flagella and an ability to swim. Dinoflagellates form two different types of cyst – temporary cysts and resting cysts (Fig. 6.1). Here, the term 'cyst' refers to 'resting cyst' or hypnozygote. The terms 'germination' and 'excystment' are used synonymously, as are 'cyst formation' and 'encystment'.

6.1.2 Temporary cyst

This non-motile cell is formed when motile, vegetative cells are exposed to unfavourable conditions such as mechanical shock or a sudden change of temperature or salinity. They are typically round or oval-shaped protoplasts liberated by thecal rupture (ecdysis). Initially, cell contents are the same as those of vegetative cells, but through time starch grains become apparent and pigments break down and change their cellular distribution (Anderson, 1980). Temporary cysts are frequently observed in laboratory cultures, especially in stationary growth phase. They are occasionally observed in natural plankton samples, although it is always difficult to ascertain whether the cysts were present naturally, or were formed by the stresses of the sampling. When conditions again become favourable, temporary cysts quickly re-establish a vegetative, motile existence. The dormancy interval thus allows them to withstand short-term environmental fluctuations. All planktonic species can have a temporary cyst stage, and for most this stage is unrelated to the reproductive process. However, some species such as *Alexandrium hiranoi* and *Peridinium quinquecorne* use this stage for asexual reproduction (i.e. they can complete asexual cell division only through the formation of temporary cysts).

6.1.3 Resting cyst

This thick-walled, highly resistant stage is occasionally formed in cultures and routinely occurs in natural plankton populations, often towards the end of a bloom (Lewis *et al*., 1979; Anderson *et al*., 1983). Resting cyst formation (Fig. 6.1) begins with the sexual fusion of gametes, which produce a swimming zygote (planozygote) that remains in the plankton for several days before falling to the sediment as a non-motile cyst (termed a hypnozygote). Under favourable conditions, some cysts can remain viable in sediments for five to ten years, sometimes even longer.

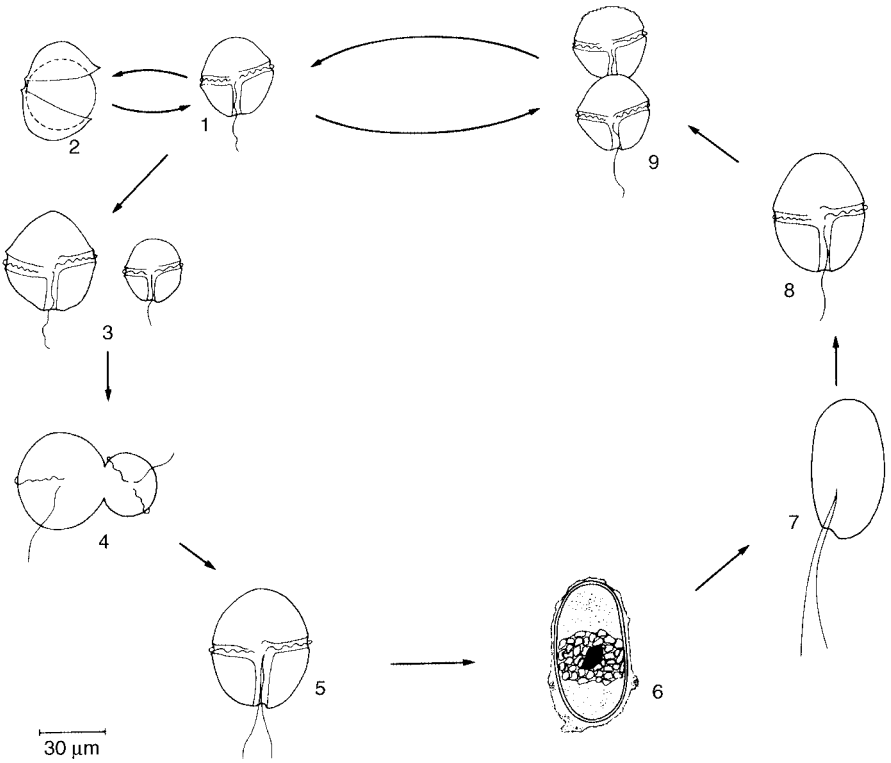

Figure 6.1
Life-cycle diagram of a dinoflagellate, using *Alexandrium tamarense* as a model. Stages are identified as follows: 1, vegetative, motile cell; 2, temporary or pellicle cyst; 3, anisogamous 'female' and 'male' gametes; 4, fusing gametes; 5, swimming zygote or planozygote; 6, resting cyst or hypnozygote; 7, 8, motile, germinated cell or planomeiocyte; 9, pair of vegetative cells following division.
Source: Anderson (1998).

6.1.4 Dormancy *v.* quiescence

It is important to use dormancy terminology with care. The literature on seeds of higher plants defines 'dormancy' as the suspension of growth by active endogenous inhibition, and 'quiescence' as the suspension of growth by unfavourable environmental (i.e. exogenous) conditions. Thus dormant cysts cannot germinate, even under optimal environmental conditions, while quiescent cysts are competent to germinate, but are inhibited from doing so by some environmental factor. Most cysts must proceed through a mandatory resting period (lasting weeks to months, depending on species) before they are capable of germination. This interval is generally considered a time for physiological 'maturation' (Pfiester and Anderson, 1987). The length of this mandatory interval varies considerably among species (12 hours to six

months: Pfiester, 1977; Anderson, 1980), and for a single species, can vary with the storage temperature as well. Thus cysts of *A. tamarense* stored at 4°C mature in four to six months, whereas storage at warmer temperatures shortens the mandatory interval to two months or less (Anderson, 1980). The duration of this process can have a significant effect on the timing of recurrent blooms, as species with a long maturation requirement may only seed one or two blooms per year, whereas those that can germinate in less time may cycle repeatedly between the plankton and the benthos and contribute to multiple blooms in a single season. Recent studies, however, suggest that some species such as *Gymnodinium catenatum* and *Pyrodinium bahamense* may not require this maturation period (Blackburn *et al*., 1989). Once a cyst is mature and the dormancy interval is over, the resting state will continue if external conditions are unfavourable for growth. Thus a quiescent cyst cannot germinate until an applied external constraint (such as cold temperature) is removed. A further complication arises in species that can alternate between dormancy and quiescence through time, due to an endogenous annual 'clock' which restricts germination to a particular time of the year (Anderson and Keafer, 1987). Mature, quiescent cysts of *A. tamarense* did not germinate in a consistent manner when exposed to optimal growth conditions throughout the year, but instead showed a variable response depending on the season. An endogenous annual clock was implicated, which might explain the germination of cysts deposited in deep waters where seasonal environmental cues such as temperature or day length are small or non-existent.

6.1.5 Factors controlling quiescence

The factors that initiate germination of mature cysts are not known for all species. The primary stimulus for excystment of temperate species is generally accepted to be a shift in temperature to favourable levels, as occurs in seasonal warming or cooling (Huber and Nipkow, 1922, 1923; Anderson and Wall, 1978; Anderson and Morel, 1979). Spontaneous germination of cysts without a change in temperature has been noted on several occasions, however (von Stosch, 1973; Pfiester, 1975, 1977; Binder and Anderson, 1987). Cysts stored at cold temperatures often remain quiescent until the temperature is increased (Huber and Nipkow, 1922; Anderson, 1980). A similar phenomenon has been observed for cysts of *A. tamarense* held at high temperatures, which maintained quiescence for a year and germinated only when temperatures decreased to a favourable level (Anderson and Morel, 1979; Anderson, 1980). This argues for the existence of a permissive temperature 'window' within which quiescent cysts will germinate, but outside of which they will continue their resting state (Dale, 1983; Pfiester and Anderson, 1987). In general, temperature can maintain quiescence for extended periods, determine the duration of dormancy after cyst formation, synchronize or entrain cyst populations for more uniform germination, and initiate the excystment process (reviewed in Pfiester and Anderson, 1987). Temperature is thus very important in the dynamics of dormancy, quiescence and germination, although much of the research to date has focused on temperate species. Species from tropical waters where temperature fluctuations are less dramatic might not be as reliant on temperature cues, but this awaits further research.

The effects of other environmental factors on dormancy and excystment are less studied. Nutrient concentrations and other water chemistry variables are not thought to exert significant influence on germination in dinoflagellate cysts, although Binder

and Anderson (1987) report that germination of *Scrippsiella trochoidea* cysts was significantly slower in unenriched medium compared with nutrient-replete medium. However, large numbers of cysts often remain in the sediments even though ambient temperatures are suitable for excystment and cell division (Anderson *et al*., 1983). This is attributed to the burial of many cysts beneath the oxygenated surface layer of sediments. All species tested so far (Anderson *et al*., 1987) have an absolute requirement for oxygen during germination. Cysts that are buried deep in the sediment can thus remain quiescent for years, their fate either being eventual death if anoxia persists, or germination should they be transported to the sediment surface or overlying water where oxygen is available. Some species must be exposed to light for either brief (Binder and Anderson, 1986) or prolonged intervals (Anderson *et al*., 1987) before excystment is possible, but many will germinate in darkness. Light can accelerate the time for germination for these species, but it is not an absolute requirement.

6.1.6 Cyst identification

More than 81 marine and 20 freshwater species of modern dinoflagellates are known to produce cysts (Matsuoka *et al*., 1989*a*; unpublished data). Of these species, less than a dozen have been known to cause red tides or toxic episodes (Table 6.1). Some cysts are similar to their motile form, but many are completely dissimilar. The important features used in identification of cysts are the shape of the cyst body and ornaments, wall structure and colour, and the type of archeopyle (excystment opening) through which the germinated cell emerges. The archeopyle is a very useful criterion for classification to the family and genus levels. It is not visible, however, before excystment, so it is not possible to use this characteristic for identification of living cysts. Furthermore, in comparison with the morphology of motile cells, cysts are usually relatively simple, mostly spherical in shape. As a result, identification of cysts based on a single morphological character is not always reliable, and other characters such as morphology of ornaments, wall structure, colour, and paratabulation must be examined. Descriptions and photographs of the cysts of harmful algal species are to be found elsewhere in this volume. For further details, a diagram of archeopyle types and several keys based on cyst shape and archeopyle type can be found in Matsuoka *et al*. (1989*a*) and in Chapter 20 of this *Manual*.

6.2 Field studies

6.2.1 Cyst distribution (mapping)

Knowledge of the distribution and abundance of cysts can be very useful in ecological and monitoring studies. Historically, such studies have been used to define the geographic range or bloom dynamics of a particular harmful species (e.g. Anderson *et al*., 1982*a*, 1982*b*; Imai *et al*., 1991), to identify potential 'seedbeds' for bloom initiation (Tyler *et al*., 1982) or sites for monitoring (Anderson *et al*., 1982*b*), or to study the dispersal of an organism from one region to another (e.g. Anderson *et al*., 1982*b*; Imai *et al*., 1991; Tyler *et al*., 1982). In some cases, it is useful to assess only the presence or absence of a species in a sample, whereas in other situations, a quantitative estimate of the abundance of that species is needed. Clearly, the methods used for these two different determinations will differ. There is probably limited

need for quantitative cyst surveys in the initial phases of most projects. Time and effort would be better directed towards large-scale surveys on a presence versus absence basis rather than quantitative studies over a necessarily much smaller area. Once the baseline surveys are established, it might then be appropriate to monitor cyst population dynamics using quantitative methods.

6.3 Sample collection

6.3.1 Site selection

Cysts, as non-motile cells, will settle from the water column and accumulate in areas where lighter sedimentary materials such as silt and clay predominate. High-energy environments are characterized by coarse, sandy substrates, which generally have low cyst abundance due to the winnowing away of finer materials. The best sites for cyst collection are thus those where the sediment is muddy rather than sandy. Sediment maps that indicate silt and clay areas can be used to identify good collection sites, or bathymetric maps used to identify basins or other depressions where finer materials can accumulate. Protected harbours and embayments are more likely to accumulate cysts than open coastal areas with wave and wind exposure. It is also important to avoid areas exposed to the air at low tide. Living cysts can still be found in such sediments, but viability is better from sites that remain permanently submerged.

6.3.2 Presence *v.* absence

The geographic distributions of the cysts of several species have been mapped using qualitative approaches. For example, the simple presence or absence of *A. tamarense* cysts was determined along the coast of southern New England, highlighting regions with the potential for PSP, including several that had no prior history of the problem (Anderson *et al*., 1982*b*).

When the absolute abundance of a species is not needed, a variety of sampling methods can be employed to collect sediment and analyse it for the organism of interest. These include commercial coring devices and grab samplers, as well as other devices that can be modified for sediment collection. For example, an old plankton net can be lowered to the sediment surface and slowly dragged across the bottom to collect the surface sediment layer that is often very rich in cysts. In shallower waters, a hand-held, manual bilge pump connected to a garden hose with a flattened funnel taped to the open end can be used to 'vacuum' the surface sediment layer into a container. Either of these two approaches can be used in shallow waters from small boats or even while wading. If a grab sampler is used, care must be taken that the surface layer is not lost during retrieval, as it contains cysts formed in the recent past. Deeper in the sediment, cysts might be years or even decades older.

Boats are not always necessary. Careful site selection may permit access from land with chest waders. This is one way to increase the number of stations sampled for a given period of time or for a fixed budget. Another useful non-quantitative sampler is an electric or battery-powered submersible pump that can be lowered to the bottom and used as a vacuum. Be aware that pebbles and debris can clog the pump. SCUBA divers are also useful, but this requires more personnel, is depth-limited, and takes more time.

Clearly there is no standard method for collection. In shallow areas where boats cannot be used, the bilge pump is probably the method of choice. In deeper areas, a plankton net or grab sampler should be used when there is no winch, or when possible, collect several gravity cores and combine surface sediments (i.e. the top few centimetres that can be swirled and poured off easily).

6.3.3 Relative abundance surveys

Another survey approach is to count all cysts in a given sample, with each species then reported as a percentage of the total number. With this approach, it is not necessary to standardize the volume of sediment analysed in each sample, so sampling and processing methods are considerably simpler than the quantitative approach described below. This method will give a good indication of where potential cyst beds are, especially if the sediments being sampled are fairly consistent with respect to cyst deposition dynamics. For comparisons among vastly different depositional sites over long distances of coastline, relative abundance can provide a useful measure of the importance of individual species in the plankton.

6.3.4 Quantitative surveys

Comprehensive, quantitative surveys of living cysts have been conducted for *Alexandrium* species in the Gulf of Maine (Anderson and Keafer, 1985), the Bay of Fundy (White and Lewis, 1982), and the lower St Lawrence estuary (Turgeon *et al*., 1990; Cembella *et al*., 1988). Other distributional studies include those for *Gyrodinium uncatenum* (Tyler *et al*., 1982), *Gonyaulax polyedra* (Lewis *et al*., 1979), and *Chattonella* (Imai and Itoh, 1988). The distribution and abundance of cysts have been shown to correlate with bathymetric features (e.g. basins), with the fine clay and silt sediment fractions, and with the transport pathways of major current systems (Anderson and Keafer, 1985; Cembella *et al*., 1988).

Collection methods for studies in which the absolute number and distribution of living cysts are to be determined place restrictions on the type of sampling to be conducted. The samples must be intact and undisturbed, and this typically calls for gravity or box corers. Some grab samplers can obtain samples without loss of the surface layer, but this requires special precautions and should be verified before quantitative studies are initiated. Simple gravity corers can be manufactured at low cost, such as the one in Fig. 6.2 developed by the Tokyo University Fisheries Oceanography Laboratory (TUFOL). Some workers prefer to use corers with larger tube diameters than the TUFOL design (e.g. 5 cm rather than 2.2 cm). At the other extreme, a hydraulically damped Craib corer (Baxter *et al*., 1981) obtains excellent samples (Anderson and Keafer, 1985), but is quite expensive and can only be deployed from larger vessels. Box corers obtain large (0.1 m^2) samples that can be subsampled on deck by manually inserting small core tubes, but again the device is expensive and very heavy, and thus is restricted to larger vessels.

Once core samples are obtained, they should be capped (filled with water above the sediment), and stored in the dark and cold before processing. For temperate species at least, these precautions will minimize germination and bioturbation artefacts, and allow sample processing to be delayed until the samples can be returned to the laboratory.

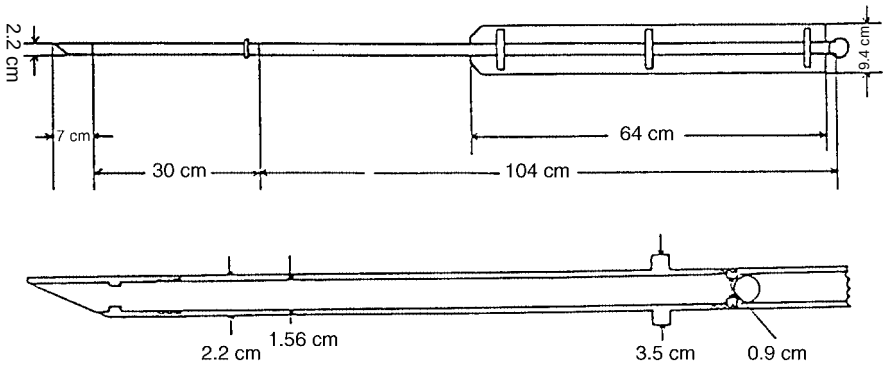

Figure 6.2
Simple gravity coring device designed by the Tokyo University Fisheries Oceanography Laboratory.
Source: Matsuoka and Fukuyo (2000).

6.3.5 Fixation

As it is often possible to store living cysts for years in the laboratory, fixation is not commonly employed. In addition, fixation often alters the appearance of the cyst contents, removing one of the diagnostic criteria that are useful in their identification. Nevertheless, long-term records of cysts are useful, and preservation is therefore needed. Buffered formalin is often used for this purpose. Formalin (37% formaldehyde) saturated with carbonate or borate is added to sediment samples to a final concentration of 5–10%. If the sample is subsequently sieved and processed as described below, additional formalin is needed, although at a lower concentration (2–3%).

6.3.6 Storage

Resting cysts are highly resistant cells that can survive burial in sediments and sometimes ingestion by animals. Cyst longevity, which probably depends most critically on temperature and oxygen, varies considerably between species, but can be at least five to ten years in natural marine sediments (Keafer *et al.*, 1992). Freshwater species are known to have survived for 17 years in lake sediments (Huber and Nipkow, 1922, 1923). Optimal survival appears to be associated with temperatures that are cold relative to the growth requirements of a particular species. For temperate species, storage at 2–4°C is desired. Storage of tropical species is problematic, as it is not yet clear how well they survive low temperatures. Refrigeration at 4°C may be harmful to tropical forms, so the best approach is probably to maintain the ambient temperature and to rely on anoxia to retard germination in stored samples. Storage of sediment samples in tightly sealed containers quickly results in anoxic conditions due to organism respiration and the chemical oxygen demand of the sediments. Anoxia is effective in maintaining quiescence without excessive mortality (Anderson *et al.*, 1987). If the sample is not sealed and is instead left open to the atmosphere, oxygenation of surface sediments can lead to germination of cysts in upper sediment layers.

In natural waters, cysts that are formed at the end of a bloom settle into the sediments and remain dormant, but viable, for months to years. Cultured cysts, on the other hand, settle to the bottom of a test tube or flask where they are exposed to conditions that are quite different from those in sediments. It is typically not possible to maintain cultured cysts for periods of time longer than one year when stored in the tube or flask in which they were formed, as many cysts die under such conditions. To extend the life of cultured cysts for long-term studies, a simple storage system has been devised (Anderson, unpublished data) in which a cyst pellet and a small amount of overlying medium are placed in a 2 ml cryovial which has a 4 mm diameter hole bored into the screw cap. This cap is secured around a piece of small-pore nylon mesh or a flexible filter membrane (<5 µm), trimmed so that the membrane is sandwiched between the cap and the external threads of the cryovial. The vial is then submerged in a 250–500 ml wide-mouth glass jar filled with fresh, anoxic, natural sediment and a small overlying layer of water. Multiple vials can be stored in a single jar of anoxic mud. This jar is tightly capped and placed inside double Ziploc bags with the innermost bag containing about 100 ml distilled water. Dehydration of the sample, which can occur during long-term storage, is minimized in this manner. The bagged jar can then be placed in a small lightproof cardboard box and stored at the desired temperature. The porous membrane in the cryovial cap allows the conditions in the vial to acclimate to those in the outside sediment, allowing cultured cysts to remain viable for several years.

6.4 Sample processing

Two basic procedures are used to process sediment samples for dinoflagellate cysts. One used predominantly for living cysts involves sieving (and sometimes sonication), but no harsh chemicals. This was originally described by Wall and Dale (1968). The other is a chemically rigorous palynological technique which leaves only cyst walls intact (see Dale, 1979). A separate procedure useful for cysts of *Chattonella* and other species is also given below.

6.4.1 Sieving technique (non-quantitative)

1. Prepare a series of sieves of various mesh sizes. These can either be commercial metal sieves employed in sediment grain-size analysis, or hand-made, inexpensive units (short, open segments of PVC pipe with Nitex mesh epoxied to one end). Useful sizes are 250 µm, 125 µm, 80 µm, 38 µm and 20 µm. Choose a suitable series of sieves for the sediments being processed and the species of interest. For example, a 250 µm sieve is not needed unless sediments contain many broken pieces of shell or plant tissue. For cysts of small species (i.e. *Alexandrium* or *Scrippsiella*), a 20 µm sieve is used in the final step to collect the particles of interest; for larger species, a 38 µm sieve is used. Usually, only two sieves are needed, one to remove large debris and the smaller to collect the particles in the size range of interest.
2. Take 5–10 ml of sediment slurry and add filtered seawater (FSW). This slurry can then be sonicated or not, depending on the objective of the study. If cyst enumeration is the only goal, sonication is recommended, as it disaggregates particles and frees cysts from other detritus. Sonication prior to physiological

studies is potentially problematic, as it is not known if the ultrasound has effects on cyst physiology. When clean, detritus-free cysts are required for a study, sonication is the only alternative. In such cases, careful temperature control using a water bath during sonication is recommended to eliminate heating effects. Probe sonicators are preferable to bath-type units due to their higher energy, although the latter will work if samples are small and are left in the bath for extended periods (5–10 min, minimum). There are many different brands of sonicator, so the necessary processing time varies. Start with 30–60 seconds at a moderate setting with probe units. This can be adjusted depending on results.

3. The sonicated (or unsonicated) sample is then poured slowly through the largest sieve (typically 125 µm or 80 µm) and the filtrate collected in a beaker. The sieve is rinsed thoroughly with FSW, and all the liquid that was collected in the beaker is poured through the smallest mesh (typically 38 µm or 20 µm). This liquid will go through more slowly than with the larger mesh, but flow can be increased by tapping the sieve with your fingers from below, blotting on paper towels, or spraying with FSW. The material on the sieve is then washed into another beaker, Petri dish, or vial. The final dilution of sediment into the vial with the FSW should be light grey in colour. 5–10 ml of slurry optimally gives about 10 ml of suspension for analysis.
4. There are several methods for separating the cysts from heavier materials. One is to put the final suspension in a small beaker (25–50 ml) and then to move the beaker in continuous small circles on the bench top. This creates a vortex in the sample, which collects sand in the centre and keeps cysts and lighter material in suspension. A pipette can then be used to remove the bulk of the liquid while the swirling continues, leaving behind a residue of sand to be discarded. Alternatively, if the sample is placed in a Petri dish, FSW can be squirted at one side of the dish using a wash bottle in such a way that the residue is surrounded by the swirling motion of water. Cysts and other light particles will be suspended in the circulating water while heavy sand particles remain at the bottom in the centre of the Petri dish.

6.4.2 Sieving technique (quantitative)

Quantitative methods for living cysts in sediment cores are detailed in Anderson *et al*. (1982*a*) but are summarized below for convenience. The following procedure applies to intact sediment cores.

1. Set up a ring stand near a sink or source of running water and stabilize with duct tape, C-clamps, or a lead weight at the base. Place a bucket in the sink for the discarded sediment.
2. Place the core tube in the ring stand, remove top core cap, and then carefully remove the overlying water by aspiration. Be sure that the core is undisturbed and that any surface disturbances have settled before aspirating. Suction all the water, removing as little of the flocculent surface layer as possible.
3. Make an extrusion stopper – typically a rubber stopper that is cut horizontally so that it fits snugly inside the core tube. It should be snug enough to support the weight of the overlying sediment without sliding down the core tube, yet loose enough to allow the sediment to be pushed upwards from below. Place the extrusion stopper on the laboratory bench.

4. Remove the bottom core cap and very quickly push the bottom of the core tube on to the extrusion stopper while pushing down on the core tube. Place an extruder against the bottom of the core tube and push the sediment up until the top edge of the sediment is even with the top edge of the core tube. The extruder is any cylindrical device that is smaller than the diameter of the core tube that can be used to push the extrusion stopper upwards. A smaller diameter, but longer (capped) core tube works well.
5. Wipe any moisture off the core tube and mark, with a waterproof pen, the intervals to be sectioned. Typically the core is cut into 1 cm or 2 cm slices, 6–12 cm deep into the core. Start by making the first mark at a convenient and visible reference point on the core tube, usually the interface between the extruder and the bottom of the extrusion stopper. Using a metric rule, mark off the appropriate intervals up to the desired depth or until you reach the top of the core tube.
6. To subsample the first interval, which is usually very liquid, push the fluid sediment up a few millimetres. Place a beaker along the top edge of the core tube, then scrape the sediment into the beaker with a large spatula. Repeat until you have reached the interval mark (i.e. 1–2 cm) as designated by lining up the interval marks on the core tube with the reference point (i.e. the extrusion stopper/extruder interface). Do not push too far at any one time or the sediment will run down the side of the core tube, losing part of the sample.
7. Using a small spatula, mix and then subsample from the sediment in the beaker. Measure the volume of the mud (usually 5 cm^3) in a cut-off syringe, eliminating all air pockets. The spoon end of the spatula works well to load the syringe and the flat end is used to scrape off the excess. Dispense the mud into a sample container. Rinse the syringe with FSW into the sample container and then rinse the walls of the sample container. Label the container with sample location, date, depth interval, etc.
8. Rinse the beaker, spatulas and syringe with tap or seawater to prepare for the next interval. Push the sediment up another 1–2 cm with the extruder. The deeper the core is penetrated, the more compact the sediments become and therefore the less it is necessary to push the sediment up in small increments to complete the interval. Due to wall friction, the lower sediment layers become contaminated by the upper layers during the coring and extruding process. The outside edge of the sediment core should thus be trimmed and discarded (a few millimetres). This is best accomplished by slightly loosening the clamps on the ring stand, allowing the core tube to be rotated while cutting the outer edge of the sediment. Scrape the remaining sediment into a dry beaker and subsample as in step 7.
9. Repeat step 8 until all the intervals are subsampled. Again, the sequence is to push the sediment up, trim and discard the outer edge, scrape the sample into a beaker, mix the contents of the beaker, and subsample using the small spatula and syringe.
10. Make sure that all the samples contain adequate seawater so that they will not dry out. Store them at an appropriate temperature (usually 2–4°C for temperate species) in the dark.

6.4.3 Concentrating cysts

For both techniques mentioned above, one constraint is that sieved material predominantly consists of sediment and detritus. Unless cyst concentrations are high, enumeration and isolation are very time-consuming. In addition, it is difficult to state with conviction that a given area is free of cysts as the methods and time constraints typically limit the amount of sediment actually examined to approximately 1 ml. Techniques have thus been developed to concentrate cysts from both sediments and laboratory cultures. The Percoll seawater density gradient procedure which has been used to separate live meiofauna and microfauna from sediments is not suitable for dinoflagellate cyst concentration because the maximum density of the medium is about 1.15 g cm^{-3}, which will not 'float' all cysts. The metrizamide gradient method used by Anderson *et al*. (1985) has the correct range of densities for cysts, but is expensive for large-scale studies. A new method was thus developed for separating and concentrating cysts using a non-toxic, aqueous colloidal silica suspension called Nalco 1060 (Schwinghamer *et al*., 1991). The procedure is summarized as follows.

1. Prepare a 'light' and 'dense' solution of Nalco. The light solution should be 22.46% (wt/vol) sucrose in distilled water, buffered to pH 8.1 with 0.0125 M TRIS (hydroxymethyl aminomethane) plus 0.0125 M TRIS HCl (final concentration). The dense solution should be a 50% (wt/wt) suspension of colloidal silica (Nalco 1060; Nalco Chemical Co., Chicago, Ill.) and aqueous sucrose at a final concentration of 11.23% (wt/wt), buffered to pH 8.1 with 0.0125 M TRIS plus 0.0125 M TRIS HCl.
2. Prepare either linear gradients (using a gradient mixer) or a step gradient in a 50 ml centrifuge tube using the two solutions. A step gradient is formed by placing a 5 ml cyst suspension in a 50 ml centrifuge tube. The 20 ml of light solution is carefully injected underneath the sample, thereby displacing it upwards. In the same manner, 20 ml of dense solution is pipetted beneath the light solution.
3. If a linear gradient is used, carefully underlay a 5 ml sediment slurry (or culture cyst suspension) below 40 ml of gradient. A pipette works well if care is taken not to disturb the layers.
4. Balance the tubes by adding or removing liquid at the surface.
5. Centrifuge at 3,000 rpm (~ 1,600 × g) for 30 min at 4°C.
6. With a linear gradient, withdraw centrifuged material in 5.0 ml aliquots using either the device described by Schwinghamer *et al*. (1991) or careful pipetting. One or at most two aliquots should contain a clean suspension of cysts. If a step gradient is used, cysts should be found at the interface between the light and dense solutions.

An alternative to physically concentrating cysts to facilitate detection or enumeration is to make the cysts themselves more visible so that they stand out against a background of detritus and other organisms. One approach recently developed by Yamaguchi *et al*. (1995) relies on the fluorochrome primuline, which binds to the outer wall of *A. tamarense* cysts. When viewed with an epifluorescence microscope, the primuline-labelled cysts exhibit an intense yellow-green fluorescence under blue-light excitation. This makes it easy to find and enumerate *A. tamarense* cysts in a sediment sample. The method also stains other dinoflagellate cysts, but the fluorescence characteristics are sometimes different. The dark walls of *Protoperidinium* cysts, for example, do not fluoresce well following primuline staining.

6.4.4 Palynological processing

The technique introduced here is mainly based on standard palynological processing (Matsuoka *et al*., 1989*a*). It uses several dangerous chemicals and therefore should be undertaken only with adequate safety precautions.

1. Place 1–2 ml of sediment into a 15 ml polyethylene test tube (do not use glass). Centrifuge and wash with distilled water several times to remove salt.
2. Add 5% hydrochloric acid to the tube to remove calcium carbonate from calcareous nanoplankton, foraminifera and other organisms. The calcareous cyst wall and ornaments such as on *Scrippsiella* and *Ensiculifera* will also be removed at this time, but the inner organic phragma will remain.
3. Centrifuge and wash with distilled water.
4. Append 1% potassium hydroxide solution to the tube and warm to 70°C in a water bath for 3 min. At higher temperature and with longer heating, the relatively thin phragma of *Protoperidinium* and *Alexandrium* sometimes disappear.
5. Centrifuge and wash with distilled water, then add concentrated (25–30%) hydrofluoric acid to the tube to remove silicate materials such as sand, diatoms and silicoflagellates. Warm in the water bath at 70°C for 2–3 hours. As the hydrofluoric acid is very dangerous and toxic, this processing should be carried out in a hood with rubber or vinyl gloves. The residue solution containing hydrofluoric acid should be neutralized with calcium carbonate.
6. Centrifuge and wash with distilled water. When cellulosic materials such as plant tissue are abundant in a sample, acetylation may be useful to remove them. The acetylation procedure is as follows:
   (a) Add glacial acetic acid to the tube.
   (b) Centrifuge and wash using Erdtman's solution, which is a mixture of nine parts acetic anhydride and one part concentrated sulfuric acid.
   (c) Warm the tube in a water bath at 70°C for 15 min.
   (d) Remove the Erdtman's solution and add glacial acetic acid again.
   (e) Centrifuge and wash with distilled water.
7. Prepare a series of sieves of various mesh-sizes with 250 µm being the upper sieve, 125 µm in the middle and 20 µm at the bottom.
8. Pour all the residue on to the upper sieve and wash it thoroughly. Cysts and other organic particles such as spores and pollen grains will pass through 250 µm and 125 µm sieves and accumulate on the 20 µm sieve.
9. Wash the remaining material on the 20 µm sieve into a 20 ml vial using distilled water for a final volume of 10 ml.

6.5 Cyst enumeration

For presence versus absence surveys, the absolute number of cysts of a target species is not as important as the fact that the species is present in a sample. Thus the objective is to scan sufficient material to make this determination, without devoting so much time that the survey becomes unrealistic. To be thorough, 3 ml or more of the final suspension should be examined in a 1 ml capacity Sedgewick-Rafter slide or its equivalent before a negative finding is noted. Scanning at 100× or 160× total magnification is most efficient. If a cyst is found, this should be verified by looking for more. A good quality microscope is very important in this process. Poor objectives

or illumination, or even poor sample-processing (typically insufficient sonication), can make many copepod eggs and pollen grains look like cysts. Cysts may even need to be isolated for closer examination under higher power, as the Sedgewick-Rafter slide often cannot be used with objectives above 20× magnification. Even those skilled in cyst identification will require 20 min to 30 min to scan a 1 ml slide, therefore 1.5 hours may be required for one sample (2 hours if processing is included).

For quantitative studies, the objective is to obtain an accurate estimate of the cyst abundance in a sample. This often requires determination of the horizontal and vertical cyst distribution (Anderson *et al*., 1982*a*; Dale *et al*., 1999). Vertical profiles of cysts within the top 6–10 cm of a core are useful in ascertaining the number of cysts that are near the oxygenated surface layer where germination is possible, as well as the total number of living cysts in a sample. Even deeper cores are sometimes used when the long-term history of cyst deposition is sought, such as in studies of species dispersal into an area (Keafer *et al*., 1992) or of environmental changes in an area (Dale *et al*., 1999). Horizontal cyst maps are useful in delineating the population distribution in an area or in pinpointing potential seedbeds (Tyler *et al*., 1982; White and Lewis, 1982; Anderson and Keafer, 1985). The most useful approach is an aerial contour map, which smoothes out small-scale irregularities in the cyst distribution and provides a good image of the overall cyst distribution in surface sediments. To accomplish this, it is necessary to arbitrarily select a depth interval over which the cyst abundance is to be tabulated. Given that marine sediments are typically anoxic below the top centimetre, one approach would be to tabulate and plot only those cysts in the oxygenated surface layer. However, this ignores the majority of the living cysts at a station, and does not account for re-suspension of deeper cysts by storms, fishing or bioturbation.

Once the core has been processed as described above, cysts can be enumerated in any counting chamber as long as it holds sufficient volume. A 1 ml Sedgewick-Rafter slide is often used, but if cysts are abundant, a 0.1 ml Palmer-Maloney slide might suffice. Care should be taken to mix the cyst suspension well before subsampling, and to distribute the sample evenly over the slide. The number of cysts to count will vary with the level of accuracy desired and the time available for the study. Once the number of examined cysts is known in a given volume, the abundance in the original sediment sample can be calculated knowing the volume or dry weight of sediment processed. Results can be expressed as cysts cm^{-3} or cysts ml^{-1} of sediment, but it is also recommended to dry and weigh the sediment sample so that cysts g^{-1} can be determined.

As discussed above, cysts have been quantified in sediment samples using two different approaches. The first, based on geological and geochemical methodologies, expresses the number of cysts relative to the mass or dry weight of sediment. The second approach expresses the cyst abundance in terms of the volume of sediment (millilitres or cubic centimetres). Proponents of the dry-weight approach argue that it is not possible to accurately measure a volume of wet sediment or that sediment compaction or differential water contents can alter the true distribution if different depths within the sediment or different sediment types are to be compared. Normalizing cyst abundance to dry weight would therefore allow for comparisons across different sediment types and depths. Those who favour the volume approach to cyst enumeration point out that it is possible to accurately measure a volume of wet sediment from a core sample. Furthermore, it is known that for some sediment types, cyst profiles look identical whether expressed per volume or per gram dry weight

(Fig. 6.3), so the error associated with variable water content with depth can be insignificant depending on the situation. Additionally, the volume approach allows the cyst abundance to be expressed per unit area (i.e. cysts m^{-2}), which is a biologically meaningful term. Modellers, or those attempting to estimate the flux of cysts from surface sediments, need to know the number of cysts in a given area or given volume. It is not possible to initialize models or to estimate cyst dynamics parameters from dry-weight estimates.

Detailed studies have not yet been conducted to show the conditions under which vertical profiles or horizontal maps of cyst abundance would differ between these two types of normalization, although it is clear that in some sediments (e.g. Fig. 6.3) there is no significant difference between vertical profiles measured per unit volume or per unit dry weight. This is probably not true in all sediment types, thus the choice of quantitative method will depend on the location and objective of the study. Vertical profiles of cyst abundance used for paleo-oceanographic or paleo-environmental studies (e.g. Dale *et al*., 1999) are informative when cyst abundance is expressed on a dry-weight basis. On the other hand, efforts to study the dynamic of cyst populations will require estimates of their area or volume distribution in sediments (e.g. Tyler *et al*., 1982; Anderson and Keafer, 1985). Workers unsure of which approach to take should perhaps do both – count the cysts in a known volume of sediment, and then dry that sediment to determine the abundance per gram.

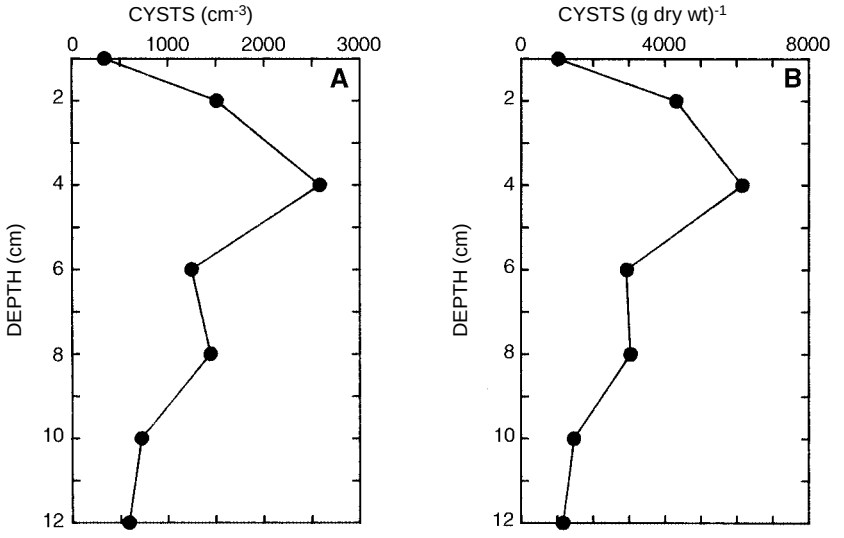

Figure 6.3
Vertical profiles of *Alexandrium tamarense* cysts in Gulf of Maine sediments, expressed (left) per unit volume or (right) by dry weight. *Source:* Anderson, unpublished data.

6.5.1 Most probable number

The most probable number (MPN) technique was initially introduced for the quantitative analysis of cysts of *Chattonella antiqua*, *C. marina* and resting spores of *Skeletonema costatum* in sediments by Imai *et al*. (1984) under the name 'extinction dilution method'. The method is useful for these and other cysts that are simple in morphology and small in size, and thus difficult to detect in sediment samples. Later Erard-Le Denn (1991) adopted this technique under yet another name for the quantitative estimation of *Alexandrium minutum* cysts. Note that numerical data obtained by this technique are not necessarily equivalent to the number of living cysts in sediments determined by sieving techniques. The MPN data give an estimate of the abundance of cysts that are capable of germinating at that time, but as some cysts might be newly formed, and thus incapable of germination (i.e. they are immature), the MPN method would underestimate the potential seed population of that species. It is important to understand the dormancy and excystment characteristics of the species being investigated if this method is to be used. A summary of this method as given by Imai *et al*. (1984) follows, with minor modifications.

1. 1 g of sediment is taken from a core or other bottom sample and sieved using 100 µm and 20 µm mesh sizes.
2. The fraction retained on the 20 µm sieve is then re-suspended in 10 ml of FSW in order to obtain a base sample in which sediment density is one-tenth of the original.
3. The base sample is diluted with culture medium as appropriate for a desired dilution series, final volume 10 ml. Dilutions of 1:10 and 1:100 are commonly used, although other options should be considered. Five replicate test tubes are then filled with 1 ml aliquots of the base and the diluted samples, all of which are then incubated under appropriate temperature and light conditions for the species under study.
4. During the incubation, the appearance of vegetative cells in the tubes is checked at four-day intervals under a microscope.
5. The number of tubes in which the vegetative cells appear are scored as positives.
6. The MPN of cysts in the sediment sample is then calculated according to the statistical table of Throndsen (1978). See also Chapter 4 of this *Manual*.

6.6 Cyst dynamics

Cysts are important during both the initiation and decline phases of blooms, so it is often desirable to study the magnitude and mechanisms of excystment and encystment. Despite the importance of such knowledge, however, it is difficult to obtain more than qualitative information about these processes, because of the complex ecological transformations that are occurring and the dynamic nature of the planktonic environment.

6.6.1 Encystment

Several approaches have been used to study the dynamics of encystment. These include the use of sediment traps, frequent observations of sediment samples, and enumeration of planozygotes in bloom populations or the relative proportions of living versus empty cysts.

Perhaps the best estimates of the flux of cysts to the sediments during a bloom can be obtained using sediment traps (Balch *et al*., 1983). However, there are no widely accepted procedures or designs, and it is known that various configurations will collect different quantities of material. Furthermore, material re-suspended from the bottom and collected in the trap may complicate the interpretation of sedimentation data. Quantitative analysis of sediment trap data is thus not recommended. It is, however, possible to learn a great deal at a qualitative level about the timing and relative magnitude of cyst formation as a component of phytoplankton population dynamics (Balch *et al*., 1983; Kremp and Heiskanen, 1999). For these purposes, sediment traps need not be elaborate or expensive. A small trap consisting of a 2 l wide-mouth polyethylene bottle attached to a line between a surface buoy and a bottom weight can be used to collect newly produced cysts. A relatively large trap consisting of three 20 l polyethylene bottles tethered together can be used to collect cysts for analyses requiring large numbers of cells, such as for toxin analysis.

6.6.2 Sediment sampling

In theory, it should be possible to repeatedly subsample an area to monitor the changes in cyst abundance as excystment and encystment occur through time. In practice, care must be taken to collect and process sufficient replicate cores at several different stations to account for the patchy distribution of cysts in the sediments and the patchy bloom populations in the overlying waters. Accurate positioning of the vessel is also necessary. In studies of this type, multiple cores are taken at each of several stations. These are sectioned and processed as described above, and the abundance of cysts in the surface layers carefully enumerated. Changes in the abundance of cysts of a species can then be correlated with the dynamics of the overlying motile-cell bloom population. Changes in the morphology of the cysts are useful as well, as newly formed cysts are sometimes easily distinguished from older, mature cysts (Anderson, 1980). For some species (see below), chlorophyll fluorescence can be used to distinguish new (and germinating) cysts from those that are dormant or quiescent.

Another approach is to identify life-cycle stages in the plankton that are indicative of sexuality and cyst formation. For many species, gametes are not easily distinguished from asexually dividing vegetative cells, but planozygotes and newly formed cysts are distinctive. Tabulation of these different stages as a percentage of the total population of a species can provide valuable information on the timing and magnitude of cyst formation. The simplest method is to use the large size and distinctive morphology of the planozygotes as diagnostic indicators (e.g. Anderson *et al*., 1983), but it is also possible to use cytological techniques to stain basal bodies and flagella to provide unequivocal evidence of the life-cycle stage of individual cells (Tyler *et al*., 1982; Coats *et al*., 1984).

Lewis *et al*. (1979) demonstrated the utility of monitoring the relative proportions of living versus empty cysts of *Gonyaulax polyedra* in studies of the dynamics of that species. Samples were collected and processed as described above, but empty cyst walls were counted as well as living, viable cysts. As cysts were formed and deposited in surface sediments, the percentage of empty cysts decreased, providing a useful indication of the timing and magnitude of cyst formation. This approach is useful only for those species that have resistant and distinctive cyst walls. Species such as *Alexandrium*, which produce nondescript cysts, would be difficult to study in

this manner as the cyst walls would be difficult to distinguish from other detritus in a sediment sample.

6.7 Excystment

Several of the methods used for studies of encystment dynamics can also be applied to excystment studies. Frequent sampling of sediments to monitor cyst abundance and vertical distribution can sometimes reveal changes associated with excystment, though again, the patchiness of cyst accumulations makes this approach difficult. Monitoring of life-cycle stage in the plankton can also be attempted, in this case the newly germinated planomeiocyte being of special interest. In dinoflagellates, planomeiocytes can be distinguished by their large size and trailing, 'ski-track' flagella, but such features are not always discernible without special scrutiny or cytological stains. One useful procedure is to use a viscous medium such as glycerol in FSW to immobilize living cells and to make their dual longitudinal flagella visible. This procedure is obviously difficult to apply to multiple, fresh, field samples. An alternative but complex approach is to use protargol staining to highlight basal bodies as diagnostic features (Coats *et al*., 1984).

Another useful technique that can provide information on the germination dynamics of some species involves monitoring the chlorophyll fluorescence of cysts. In *A. tamarense*, for example, quiescent cysts show no red fluorescence when excited with blue light until they begin to alter their physiology in preparation for germination. Cysts in surface sediments can thus be examined through time using an epifluorescence microscope to document the temporal change in the proportion of the population that is synthesizing chlorophyll. Anderson and Keafer (1985) used this approach to demonstrate the rapid germination cycle of *A. tamarense* in shallow waters, compared with the long, gradual germination process in deeper coastal waters.

Excystment rate data can be collected using the germination trap sampler of Ishikawa *et al*. (1995). The trap is an inverted, 10 µm mesh-lined pyramid that covers a known area of the bottom (0.1 m^2). A hose connection to the surface allows water inside the trap to be removed and screened for vegetative cells. Although cell growth and grazing are potential problems, daily sampling can provide useful germination estimates. Examples of this approach are in Ishikawa and Taniguchi (1996). It should be noted that this trap will be difficult to deploy in deeper waters, or in areas with very soft sediments. The mesh on the pyramid must also be selected such that it does not allow vegetative cells of the species of interest to be drawn into the trap during pumping, as these cells would be mistakenly assumed to be germlings.

6.8 Laboratory studies

6.8.1 Isolation and germination

Laboratory studies of cyst-forming species require that cultures be established. This can either be accomplished by isolating individual vegetative cells from plankton samples, or by isolating cysts and germinating them to obtain the initial cells for cultures. Isolation procedures employ micropipettes of various types. Pasteur

pipettes whose tips have been drawn out over a flame (50–100 µm diameter) are commonly used, either connected to a tube which the user sucks on to draw cells into the tip, or used alone such that capillary action draws up the cells. Another method uses capillary tubes (1 mm i.d., non-heparinized), which are drawn into very fine, hollow strands over a flame and snapped off to produce a narrow tip. These are attached to thin tubing (such as that used on many nutrient autoanalyzers). The thin tubing and very fine capillary tip restrict the amount of material that is drawn into the tube with mouth suction.

A small amount (~0.5 ml) of the sieved cyst sample is placed onto a large counting chamber such as a Sedgewick-Rafter slide. Filtered seawater is then added to 1 ml, and the cysts isolated by micropipette. If necessary, a small needle (insect pin) attached to the tip of a glass tube can be used to clear the background around a cyst on the slide.

Isolated cells or cysts are placed into culture medium suitable for the species of interest. They can be incubated in culture tubes, in the wells of tissue culture plates, in Petri dishes, or in individual slides. The 96-well tissue culture plates are useful because they can be monitored using a dissecting or an inverted microscope to determine the success of the isolations. The wells are half-filled with medium, individual cysts are isolated and deposited in the well, the cover replaced, and the entire chamber sealed with plastic tape for incubation. Plates can be easily scanned with an inverted or stereomicroscope, but this does not allow cysts to be easily pinpointed for individual observation. It does, however, make it very easy to quantify cyst germination as long as the emerging cells swim or divide.

If accurate germination statistics are needed, the Palmer-Maloney slide method of Wall and Dale (1968) can be used. The cyst (or cysts – no more than 10) are isolated from a sediment sample and placed in the centre of the Palmer-Maloney chamber with a small drop of medium, and a ring of Vaseline or silicone grease is placed around the outer edge of the raised grey surface. (Care must be taken that the grease is non-toxic.) A large cover slip is placed over this and pressed down so that the drop of medium becomes a flattened disc that remains in the centre of the slide, not touching the edges of the well or the grease. No appreciable evaporation will occur for at least one week. Individual cysts are then located and their coordinates noted using the Vernier scales found on many microscope stages. Use of the coordinates allows the user to return to each specific location to look for empty cyst walls. This becomes important when pennate diatoms and other algae overgrow the slide as contaminants. (Note that Palmer-Maloney slides are surprisingly expensive. One alternative is to cut one or two sheets of Parafilm to the size of a microscope slide. These are placed on the slide and a well is cut out of the centre.)

Some workers use 36 mm Petri dishes as a convenient and reliable vessel for germination. Filled with 2–3 ml of medium and sealed with Parafilm or similar coverings, they do not lose medium to evaporation. The entire dish can be examined under a dissecting microscope, and cysts and vegetative cells are easily recognized against the dark background. The base of the dish is sufficiently clear so that examination and photography with an inverted microscope is also possible. Once germination has occurred, cells can be isolated or left to divide for relatively long periods before transfer to larger culture vessels.

The tubes, Petri dishes, tissue culture plates or slides are placed at a suitable growth temperature under lights, with germination expected in two to five days. If the original cyst processing and isolation was clean and thorough, it is possible to

establish a culture directly from the swimming cells in the tissue culture wells, either through individual isolations or using many cells at once (if other algal contaminants are not present). It is generally desirable to establish true clonal cultures, so isolation of individual vegetative cells (one cell per culture tube or well) is usually necessary. Note that if multiple cells produced by a cyst germination are used to start a single culture, the result will not be clonal, in the strict sense of the word. This is because most cysts result from mating between male and female gametes, and the germinated cyst will produce these two genetically different mating types. A true clonal culture of a heterothallic organism should not be able to produce cysts unless it is combined with a culture of an opposite mating type.

6.8.2 Encystment studies

The ability to form cysts in laboratory cultures is a major asset to any research programme on a particular organism. Examples for marine species are found in Watanabe *et al*. (1982), Anderson *et al*. (1984), Blackburn *et al*. (1989), and Nakamura *et al*. (1990). Unfortunately, some species that respond reasonably well to culturing do not form cysts so easily. It is thus often useful to start a culture collection of numerous isolates of the species of interest, in the hope that some of them will produce cysts in sufficient quantities for laboratory study.

In laboratory cultures, depletion of either nitrogen (nitrate or ammonium) or phosphorus will often induce sexuality (e.g. Pfiester, 1975; Turpin *et al*., 1978; Anderson *et al*., 1984). This needs to be carefully controlled, however, as cessation of growth in batch cultures due to overcrowding or carbon limitation does not generally induce cyst formation. If the f/2 medium (Guillard and Ryther, 1962) is the standard, nutrient-replete growth medium for a species, reduction of phosphorus or nitrogen levels to f/40 or f/80 will often result in limitation and sexuality leading to cyst formation (Anderson *et al*., 1984).

A problem arises in efforts to determine encystment rates using laboratory cultures, as nutrients are exhausted so quickly in batch cultures that some cells begin the process of cyst formation, but do not complete it due to unfavourable conditions (Anderson *et al*., 1985; Anderson and Lindquist, 1985). The low yield of cysts (10–20%) reported in such studies is deceptive, as a large percentage of the motile population can be planozygotes that presumably would have formed cysts had conditions been suitable.

Nutrient limitation has also been implicated in sexual induction in natural populations, although no direct measurements have yet proven this inference. In fact, cyst production has been observed when external nutrients were at or above concentrations that previously supported only vegetative growth (Anderson and Morel, 1979; Anderson *et al*., 1983). The precise set of environmental cues that stimulate encystment is not well defined, and recent studies indicate that factors other than macronutrient availability (e.g. iron stress) may be involved (Doucette and Harrison, 1989). Furthermore, given the discovery of endogenous control of cyst germination for *A. tamarense* (Anderson and Keafer, 1987), the possibility of endogenous or 'clock'-regulated sexuality must also be considered.

If organisms are heterothallic (or if their mating characteristics are unknown), crosses between cultures of different isolates are necessary. In such cases, individual cultures of the strains to be crossed are grown in reduced nutrient medium and then combined. Multiple isolates crossed with each other and self-crossed will generate a

matrix of successful matings that can be used to define the mating type of each isolate (e.g. Yoshimatsu, 1984). For some fastidious species, extra precautions are necessary in medium preparation. Anderson *et al*. (1984) found that cyst yield of *A. tamarense* could be increased by minimizing precipitates and chemical contaminants during medium preparation and sterilization. Although tedious and somewhat expensive, the precautions described by those authors for glassware cleaning and medium preparation are recommended for optimal growth and cyst production.

6.8.3 Excystment studies

A number of basic physiological characteristics should be determined for the cyst of a species of interest. With respect to excystment, these include the length of the mandatory dormancy interval and the factors that regulate quiescence or that trigger germination for that species. These studies are easiest when cysts can be formed in culture, but when laboratory cultures are not available for certain species, it is still possible to obtain relatively 'clean' cyst preparations using field populations. One approach that has been successfully used on *Gymnodinium catenatum* (Anderson *et al*., 1988) and numerous other dinoflagellate species (Anderson unpublished data) involves collection of a mixed plankton assemblage using a plankton net and the resuspension of that material in FSW from the study site which has been enriched with f/2 levels of vitamins, metals and EDTA. Sometimes, f/80 levels of major nutrients are also added. The assemblage is incubated in the laboratory at the temperature of the ambient water at an appropriate day length and light intensity. Within a few days, cysts will be produced by many of the species in the sample. These are of a known age and can readily be isolated by micropipette to study morphology or germination characteristics.

Cysts of a known age can also be collected in a sediment trap (deployed underneath a bloom but sufficiently far above the bottom to avoid resuspension of 'old' cysts). The least attractive option, but one which will work if a recent bloom has produced abundant new cysts, is to collect and work with surface sediments directly. Cysts can be isolated individually for germination trials, or an alternative procedure can be attempted that utilizes unprocessed sediment. This involves the preparation of a large sample of sediment 'slurry' in FSW, which is then subdivided into numerous subsamples. The cysts of interest in five to ten of these aliquots are enumerated to obtain a statistically sound estimate of initial concentrations. The remainder of the subsamples are then incubated experimentally. The difference between the initial counts and the number of cysts remaining after incubation provides a good measure of germination success (Anderson *et al*., 1987).

Once cyst suspensions are obtained using one of the above methods, a variety of experimental manipulations are possible. Determination of the mandatory dormancy interval for cysts or spores requires that cysts of known age be stored under different temperatures and periodically exposed to optimal growth conditions to assess germination success (Anderson, 1980; Binder and Anderson, 1987; Blackburn *et al*., 1989). The permissive temperature 'window' for germination (e.g. Anderson *et al*., 1985) can be determined by incubating quiescent cysts across a range of temperatures (in the light) and assessing the percentage of germination after an arbitrary interval. As germination will be slow at low temperatures, a month or more of incubation is sometimes needed. Different temperatures can be provided by the use of multiple incubators, or through the use of a temperature gradient bar

(Watras *et al*., 1982) that is heated at one end and cooled at the other, resulting in a continuous gradient in temperature. Light requirements for germination require special handling of the cyst suspensions, as even brief exposure to low levels of light can trigger excystment in some species (Binder and Anderson, 1986). Samples can be processed in near-darkness, or using a red photographic light, although in both cases some exposure is unavoidable. Tubes can then be wrapped with different layers of screening or shaded with neutral-density filters to provide the required light variation (Anderson *et al*., 1987).

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7

Analytical methods for paralytic shellfish poisons

B. Luckas, C. Hummert and Y. Oshima

7.1 Introduction

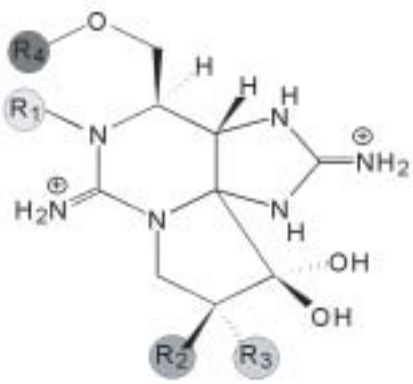
Paralytic shellfish poisoning (PSP) is caused by a group of approximately two dozen naturally occurring potent neurotoxins (Fig. 7.1). These toxins specifically block the excitation current in nerve and muscle cells, finally resulting in paralysis and other illness in consumers of contaminated shellfish (see Chapter 26). Consequently, the development of analytical methods for the qualitative and quantitative determination of the toxins associated with PSP is an important task (Luckas, 1992). In particular, the quality control of potentially contaminated seafood requires exact quantification of the PSP toxins with regard to international regulations for public health protection and international commerce (Luckas, 2000*a*, 2000*b*). The broad toxicity range of different PSP toxins (Oshima, 1995), in addition to the diverse PSP toxin profiles of dinoflagellates that cause PSP and the possibility of biotransformation of these toxins within marine organisms (Shumway, 1990; Cembella *et al*., 1994), have challenged analytical chemists to develop accurate and reliable analytical methods. Over the past few decades, considerable progress has been made in developing chemical analytical alternatives to the mouse bioassay method (see Chapter 13) for almost all the common PSP toxin analogues found in shellfish and other seafood.

7.2 Determination of PSP toxins

7.2.1 Fluorometric assay

The PSP toxins do not exhibit a strong ultraviolet (UV) absorbance or native fluorescence. A fluorometric assay method was developed based on the oxidation of the toxins in alkaline solution to fluorescent iminopurine derivatives (Bates and Rapoport, 1975). After acidification, the fluorescence intensity of the oxidation products in solution (Fig. 7.2) is measured by fluorometry in a cuvette.

The interpretation of the results of this fluorometric assay method is complicated as individual PSP toxins differ both in toxicity and fluorescence intensity after oxidation. The carbamate toxins [saxitoxin (STX), neosaxitoxin (NEO), and gonyautoxins (GTX1–GTX4)] are the most toxic, N-sulfocarbamoyl toxins (B1, B2, C1–C4) are the least toxic, and decarbamoyl (dc-) toxins have intermediate toxicity (Fig. 7.1). In general, the N–1 hydroxylated toxins (e.g. NEO, GTX1, GTX4)



Toxin	R1	R2	R3	R4	Toxicity factor (STX=1)	Molecular weight
STX	H	H	H		1	301
NEO	OH	H	H		0.92	317
GTX1	OH	H	OSO_3^-	H_2N-CO	0.99	412
GTX2	H	H	OSO_3^-	(Carbamoyl-)	0.36	396
GTX3	H	OSO_3^-	H		0.64	396
GTX4	OH	OSO_3^-	H		0.73	412
B1 (=GTX5)	H	H	H		0.06	380
B2 (=GTX6)	OH	H	H		no data	396
C3	OH	H	OSO_3^-	^-O_3S-NH-COO	0.01	492
C1	H	H	OSO_3^-	(N-sulfo-	< 0.01	476
C2	H	OSO_3^-	H	carbamoyl-)	0.10	476
C4	OH	OSO_3^-	H		0.06	492
dcSTX	H	H	H		0.51	258
dcNEO	OH	H	H		no data	274
dcGTX1	OH	H	OSO_3^-	H	no data	369
dcGTX2	H	H	OSO_3^-	(Decarbamoyl-)	0.65	353
dcGTX3	H	OSO_3^-	H		0.75	353
dcGTX4	OH	OSO_3^-	H		no data	369

Figure 7.1
Chemical structure and toxicity of PSP toxins.
Source: Oshima (1995).

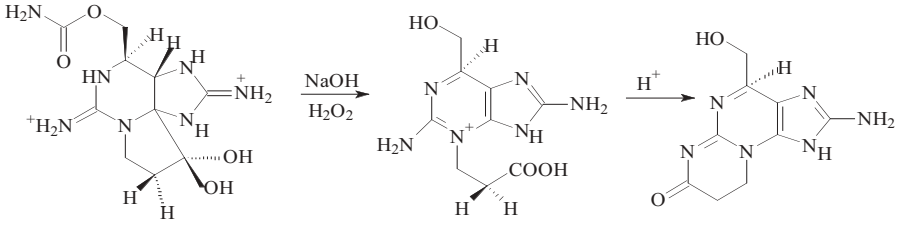


Figure 7.2
Alkaline oxidation of saxitoxin.
Source: Bates and Rapoport (1975).

exhibit a lower molar fluorescence yield than their respective analogues lacking the N–1 hydroxyl moeity (STX, GTX2, GTX3); however, no correlation exists between toxicity and fluorescence intensity (Franco and Fernández, 1993). This fluorometric assay may be sufficient for a rapid screening tool, but if the assay is to be used for quantitative purposes, independent chromatographic separation of the PSP toxins, followed by fluorescence detection of the oxidation products, is recommended.

7.2.2 Chromatographic methods

7.2.2.1 *Liquid chromatography*

It is often imperative to know which PSP toxin components are present in a sample of interest. As the oxidative fluorescence assay gives no information about individual toxins, more sophisticated analytical methods have been developed to resolve these components. Open-column (low-pressure) chromatography was used in early studies to fractionate various PSP toxin components from shellfish and dinoflagellate matrices, but now high-performance liquid chromatography (HPLC) is the most widely used analytical technique (Van Egmond *et al*., 1992), providing excellent peak resolution and high sensitivity for each toxin when combined with fluorescence detection. There are several alternative HPLC methods for PSP toxin determination described in the literature. Most are based on fluorescence detection of oxidized PSP toxins. The formation of fluorescent iminopurine derivatives can be done before or after chromatographic separation.

The importance of a complete chromatographic separation of all PSP toxins that contribute to total PSP toxicity prior to quantification with a fluorescence detector was confirmed in an intercalibration exercise. The results of this intercalibration clearly demonstrated that HPLC methods for the determination of PSP toxins were superior to the available enzyme-linked immunosorbent assay (ELISA) developed specifically for the detection of STX, and for which cross-reactivities with other PSP toxin analogues were poorly defined (Van Egmond *et al*., 1994) (see Chapter 12). Furthermore, the quantitative and qualitative data from the intercalibration exercise indicated that it was most advantageous to perform the alkaline oxidation after chromatographic separation of underivatized PSP toxins (post-column oxidation). Nevertheless, varying results obtained with different chromatographic methods for the determination of PSP toxins suggested that the conclusions to be drawn from this intercalibration exercise must be interpreted with caution (Quilliam, 1995). The HPLC methods with fluorescence detection offer good sensitivity and dynamic range for the separation and detection of the different PSP toxins. This sensitivity is dependent on parameters such as reagent concentrations, reaction time, pH, and temperature of the oxidation reaction, all factors that must be carefully controlled to achieve consistent results.

7.2.2.1.1 *Pre-column derivatization with fluorescence detection*

A liquid chromatographic method has been developed, based upon pre-chromatographic oxidation, using both hydrogen peroxide and periodic acid (Lawrence and Ménard, 1991; Lawrence *et al*., 1991, 1996). The N–1 hydroxylated toxins, NEO, B2, GTX1 and C3, form fluorescent products after periodate oxidation at around pH 8.7, but do not form fluorescent derivatives with peroxide oxidation under the specified conditions. The non-N–1 hydroxylated toxins, STX, B1, GTX2, GTX3, C1 and C2, form highly fluorescent derivatives with both peroxide and periodate

oxidation. The addition of ammonium formate to the periodate oxidation reaction greatly improves the fluorescence yield for the N–1 hydroxylated toxins (Lawrence and Ménard, 1991). The oxidation products of NEO and B2 are not separated by this method, therefore parent compounds are separated using an ion exchange cartridge before oxidation. Both periodate and hydrogen peroxide oxidations produce two fluorescent products for GTXs, dcGTXs, dcSTX and dcNEO. Neosaxitoxin cannot be determined by application of hydrogen peroxide oxidation under the specified conditions. It is still unclear how decarbamoyl toxins behave and whether or not they interfere with the quantification of other PSP toxins (Fig. 7.3).

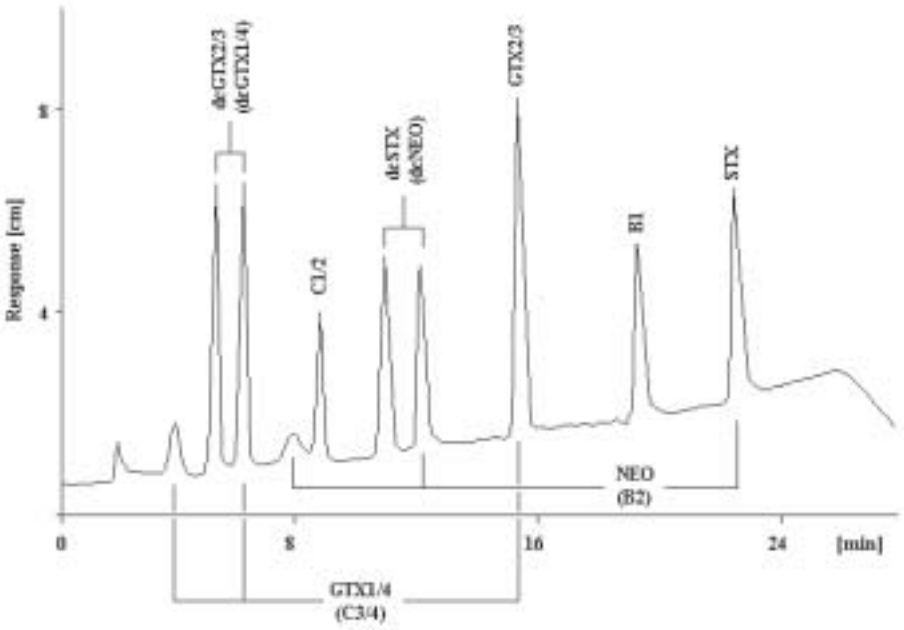


Figure 7.3
Chromatogram of oxidation products of PSP toxins.
Source: Lawrence *et al*. (1996).

7.2.2.1.2 *Post-column derivatization with fluorescence detection*

A breakthrough was achieved in the use of HPLC methods for PSP toxin determination with the successful separation of underivatized PSP toxins by ion-pair chromatography with alkylsulfonic acids, using a polymeric stationary phase (polystyrene-divinylbenzene) in the analytical column (Sullivan and Wekell, 1984). With this method, a solvent gradient is used with a phosphate buffer containing n-hexane sulfonic acid and n-heptane sulfonic acid as ion-pair reagents. Post-column derivatization is performed with periodic acid as the oxidizing reagent in a flow-through post-column derivatization unit with separate channels for oxidant and neutralizing acid. This method provides a good separation of most of the high-potency carbamate toxins in a single chromatographic run (Fig. 7.4).

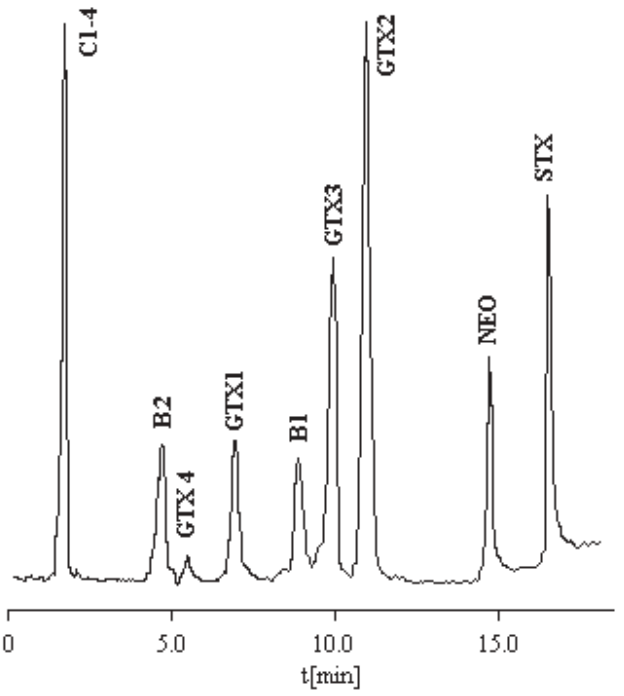


Figure 7.4
HPLC separation of PSP toxins according to Sullivan and Wekell (1984).

This separation method with a polymeric column is relatively robust, but has several disadvantages. The low potency N-sulfocarbamoyl toxins (C1–C4) are not separated and are difficult to distinguish from co-eluting fluorescent artefacts at the solvent front, and the decarbamoyl (dc-) toxins are not resolved from their respective carbamate analogues. For example, coelution of STX and dcSTX in a sample would yield an incorrect calculation of total PSP toxicity, as STX is twice as toxic as dcSTX on a molar basis (Fig. 7.1). Therefore, resolution and separate quantification of STX and dcSTX is necessary for the comparison of HPLC results with the mouse bioassay, at least where the presence of decarbamoyl derivatives is known or strongly suspected (Luckas *et al*., 1990).

Oshima *et al*. (1989) described the separation of the entire spectrum of known PSP toxins by isocratic HPLC using three separate chromatographic conditions, varying in pH and mobile phase composition: (Group 1: C1–C4; Group 2: GTX1–GTX4, dcGTX1–dcGTX4, B1, B2; Group 3: NEO, dcSTX, STX). A reverse-phase (RP) octylsilica (C_8) column is used as the stationary phase. Tetrabutyl-ammonium phosphate (Eluant A) is used for the separation of the N-sulfocarbamoyl–11-hydroxysulfate toxins C1–C4, and Eluents B and C contain n-heptane sulfonic acid as ion-pair formers for the separation of the carbamate and decarbamoyl toxins. This method suffers from the relatively high cost in time and materials per analysis because three separate chromatographic runs are required for the quantitation of all

PSP toxins in a sample. Fig. 7.5 shows the HPLC separation of the carbamate toxins with Eluent B and the decarbamoyl toxins with Eluent C, as proposed by Oshima *et al*. (1989). Since the introduction of the method, several modifications have been introduced, including those suggested by the developer (Oshima, 1995).

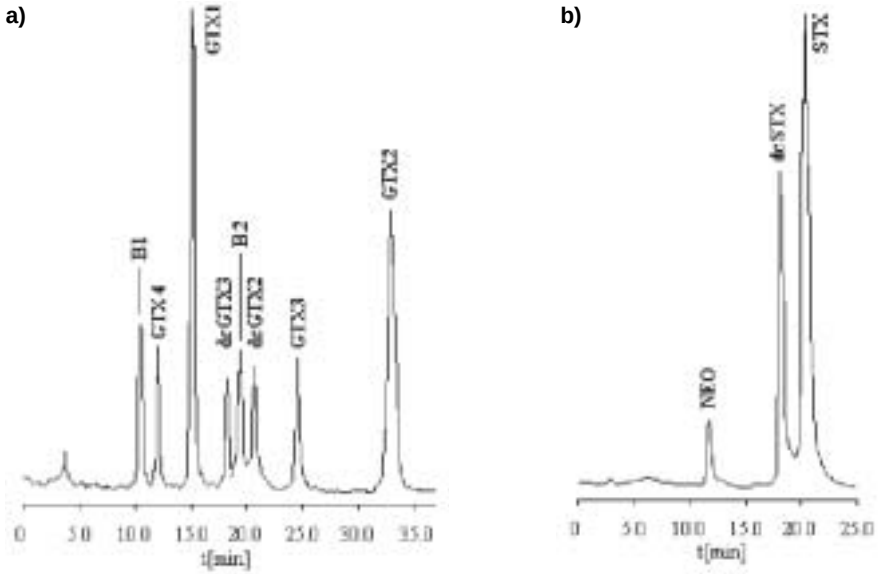


Figure 7.5
HPLC separation of PSP toxins according to Oshima *et al*. (1989): A, Eluent B; B, Eluent C.

In an alternative post-column oxidation method, Thielert *et al*. (1991) introduced ion-pair chromatography on an RP-C_{18} stationary phase, using n-octane sulfonic acid and ammonium phosphate in the eluent. With this method, isocratic elution allows the separation of STX and dcSTX, but problems arose with the separation of GTX components. A gradient with two buffers was later introduced to overcome this problem. In the current method, both eluents contain n-octane sulfonic acid, phosphate and tetrahydrofuran. The second eluent also contains acetonitrile. This HPLC method allows an extensive separation of the carbamate, decarbamoyl and N-sulfocarbamoyl toxins. Unfortunately, GTX1 and GTX4 coelute with this technique (Fig. 7.6).

To exclude inconsistencies in the determination of PSP toxins, particularly in a complex matrix with a wide spectrum of toxin analogues, and where total quantification is required, it is advantageous to apply two different HPLC methods. For example, co-application of the HPLC methods of Thielert *et al*. (1991) and Oshima *et al*. (1989) is a suitable (albeit expensive and time-consuming) solution to these problems. Fortunately, the elution order of GTX2 and GTX3 is reversed by using n-octanesulfonic acid (Thielert *et al*., 1991) instead of n-heptanesulfonic acid (Oshima *et al*., 1989). The HPLC method of Oshima *et al*. (1989) can be modified with a two-

step elution using Eluents B and C. In contrast to the method of Sullivan *et al*. (1985), no complex gradient is necessary and the analysis of PSP toxins is performed by simple switching from the first eluent to the second, which is in agreement with the method of Thielert *et al*. (1991). The joint evaluation of the chromatograms obtained with the different methods results in an accurate quantification of all PSP toxins relevant for the calculation of the total PSP toxicity in a sample.

High concentrations of phosphate and ion-pair reagent in the eluent stabilize the chromatographic system, particularly after injection of extracts containing complex matrix components. Consequently, neither baseline drift nor variations in the retention times are observed by application of HPLC methods with high concentrations of ammonium phosphate in the eluents (Hummert *et al*., 1997). Therefore, maintenance of a concentration of 40–50 mM ammonium phosphate in the buffers used as eluents in the method of Thielert *et al*. (1991) was considered in experiments directed to overcome the method shortcomings, i.e. co-elution of GTX1 and GTX4. To resolve this limitation, Hummert *et al*. (1998) developed an effective alternative HPLC method, based on the eluents used by Thielert *et al*. (1991). A different HPLC column was employed and a third mobile phase (Eluent C) was introduced. Furthermore, the pH of the eluents was optimized (to pH 6.9) and a four-step elution was proposed (Table 7.1). This method was successfully applied for determination of PSP toxins in microalgae and mussels (Yu *et al*., 1998). The toxin separation (Fig. 7.7), especially for GTXs, has been improved in comparison to the Thielert (1991) method. Furthermore, the extraction procedure, using both acetic acid and hydrochloric acid, allows comparison of results with those obtained by the mouse bioassay (see Chapter 13).

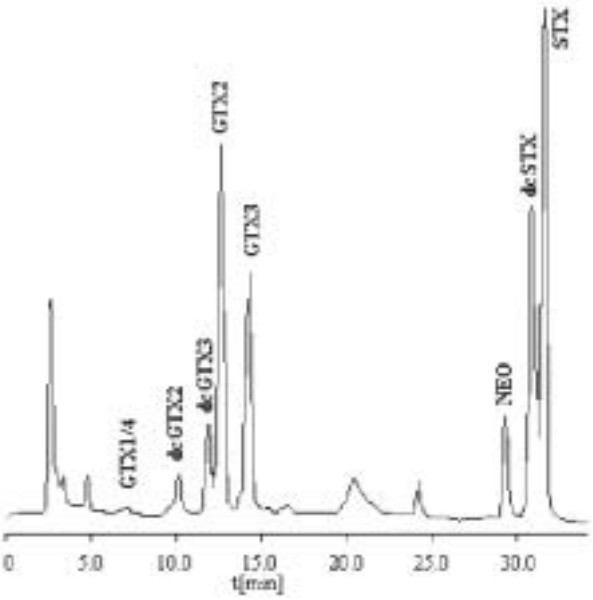


Figure 7.6
HPLC separation of PSP toxins from mussels (*Mytilus edulis*) according to Thielert *et al*. (1991).

TABLE 7.1 Determination of PSP toxins by HPLC with fluorescence detection

HPLC separation						
Column	Luna C18, (Phenomenex), 250 mm × 4.6 mm i.d., 5 µm					
Eluent A	98.5% 40 mM ammonium phosphate buffer (pH 6.9) with 11 mM octane sulfonic acid (sodium salt) 1.5% tetrahydrofuran					
Eluent B	83.5% 50 mM ammonium phosphate buffer (pH 6.9) with 15.0% acetonitrile 1.5% tetrahydrofuran					
Eluent C	98.5% 40 mM ammonium phosphate buffer (pH 6.9) with 1.5 % tetrahydrofuran					
Gradient	Step	Time (min)	Eluent A (%)	Eluent B (%)	Eluent C (%)	Flow (ml min^{-1})
	1	0.0	50	0	50	1.0
	2	13.5	50	0	50	1.0
	3	15.5	0	100	0	1.0
	4	30.0	0	100	0	1.0
	5	31.0	100	0	0	1.0
	6	45.0	100	0	0	1.0
	7	46.0	50	0	50	1.0
	8	57.0	50	0	0	1.0
Post-column oxidation						
Oxidation solution	40.0 ml ammonia solution (25%) and 1.14 g periodic acid in 500 ml water					
Acidifying solution	30.0 ml acetic acid (100%) in 500 ml water					
Reaction conditions	50°C, 1.0 min (10 m coil), flow 0.3 ml min^{-1} for both solutions					
Detection	fluorescence (Ex.: 330 nm, Em.: 390 nm)					

Source: Hummert *et al*. (1998)

The chemical oxidation of the STX ring system to form a corresponding fluorescent derivative during the post-column chemical reaction (PCRS) is very sensitive to changes in temperature, flow rate and age of the post-column reagents. Oxidation of the STX ring system can also be done electrochemically, and the use of a coulometric electrochemical cell offers both advantages and disadvantages over the more traditional chemical oxidation for liquid chromatography of PSP toxins (Boyer and Goddard, 1999). Electrochemical cells require fewer pumps and are less sensitive to changes in flow rate than the PCRS system. Electrochemical oxidation also allows for the rapid identification of naturally fluorescent impurities that may co-chromatograph with PSP toxins. True PSP toxins are not naturally fluorescent and hence their signal disappears when the oxidizing voltage is turned off. As disadvantages, the small pores in the electrochemical cells can easily become plugged and additional care must be taking in sample preparation. As with the pre-column oxidation system, excessive oxidation of NEO to multiple products, some of which

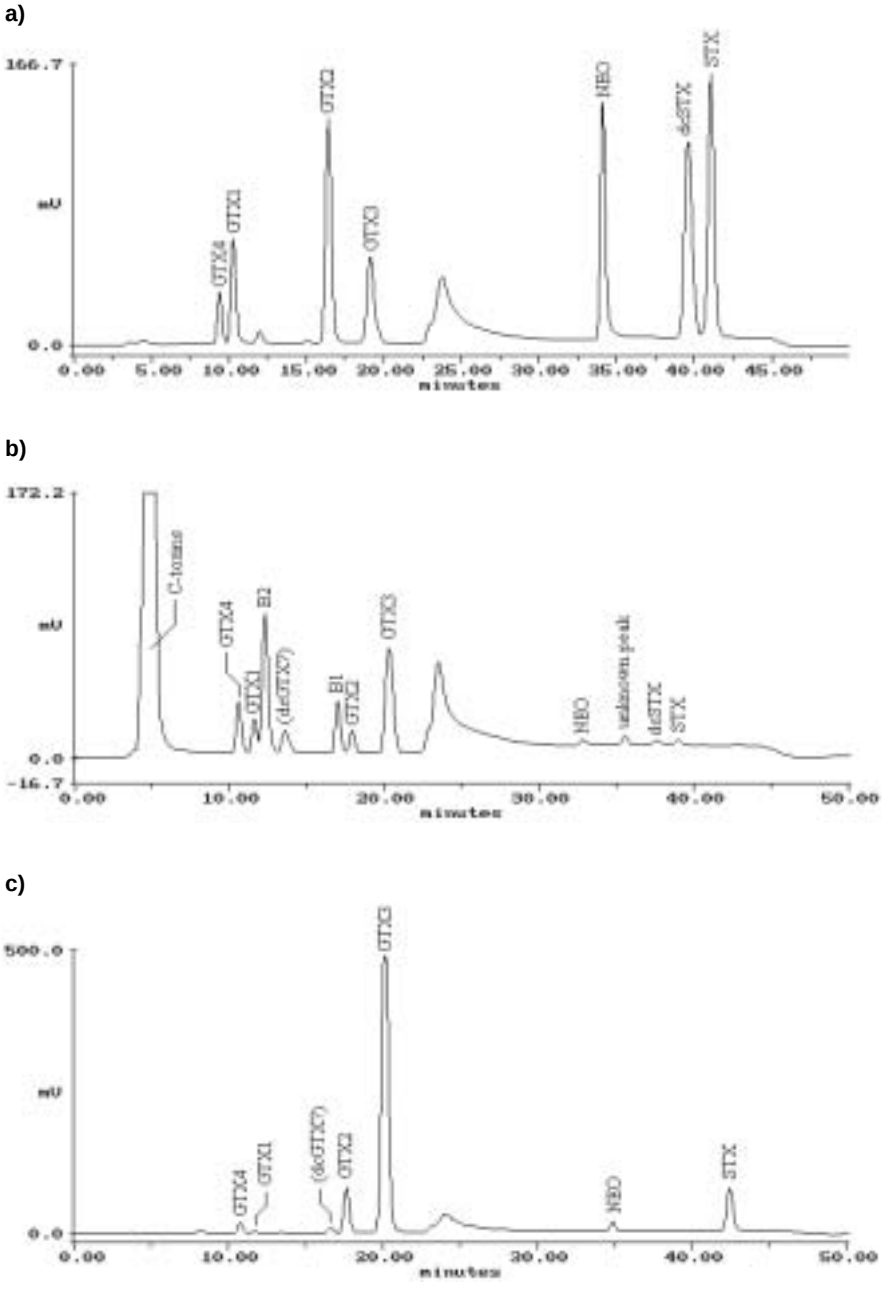


Figure 7.7
HPLC separation of PSP toxins according to Hummert *et al*. (1998): A, PSP standard mixture; B, *Alexandrium tamarense* (extracted with 0.03 N acetic acid); C, *A. tamarense* (extracted with 0.1 N HCl).

are non-fluorescent, often causes the electrochemical system to be less sensitive to the N1-hydroxy compounds, such as NEO, GTX1 and GTX4. Electrochemical oxidation is compatible with the mobile phases described previously (Sullivan and Wekell, 1984; Oshima *et al*., 1989; Hummert *et al*., 1998) with the exception of Eluant C of the Oshima (1989) procedure. As there is no post-column buffer to control the pH of the oxidation, the mobile phase must serve this purpose. Oxidation of the STX ring system does not occur under acidic conditions, hence acidic mobile phases such as Eluent C (pH 4.5) (Oshima *et al*., 1989) require the post-column addition of alkaline buffer for oxidation. A typical chromatogram of the separation and detection of NEO and STX after electrochemical oxidation is shown in Fig. 7.8.

7.2.2.1.3 *Fluorescence and mass spectrometric detection*

The use of mass spectrometry (MS) as a detection system is desirable for confirmatory analysis of samples containing PSP toxins. However, mobile-phase components, such as phosphate, ion-pair formers, etc., as well as periodic acid from the post-column derivatization unit, prevent effective LC/MS coupling. Therefore, an alternative HPLC method was developed allowing direct coupling of the chromatograph with the mass spectrometer (Kirschbaum *et al*., 1995) (Fig. 7.9). The separation of PSP toxins is achieved on a weak cation exchange resin using an aqueous eluent with ammonium acetate as the only additive.

In case of a parallel application of fluorescence (FLD) and MS detection, electrochemical post-column derivatization is suggested. Such an LC/FLD/MS system not only allows the unambiguous determination of PSP toxins, but is also well suited for the analysis and certification of PSP toxin standards (Fig. 7.10).

Recently, modification of the HPLC/FLD/MS method by adding a combination of strong anion- and cation-exchangers allowed the ion chromatographic separation of all PSP toxins relevant to seafood regulation within a single chromatographic run (Jaime *et al*., 2000). As it is possible to inject relatively large volumes into the HPLC/FLD/MS system, this method is well suited for determination of PSP toxins in biological materials, by both fluorescence and MS detection (Fig. 7.11).

By application of ion-exchange chromatography with MS detection, short-run elution is possible. Isocratic elution in combination with MS detection allows the quantification of all PSP toxins that are significant for regulatory regimes, but detection limits are higher than with fluorescence detection. However, the sensitivity of MS detection is sufficient to control seafood at the regulatory limit for PSP toxin content (800 µgSTXequivalents kg^{-1} wet weight of soft tissues) (Fig. 7.12).

7.2.2.2 Capillary electrophoresis with UV and mass spectrometric detection

Several options, other than liquid chromatography, are available for analysis of PSP toxins. Among these alternatives, capillary electrophoresis (CE) has shown some promise, although this technique is not in widespread use. A capillary electrophoresis method with UV detection was developed for the separation and determination of underivatized PSP toxins (Thibault *et al*., 1991). Separation by CE is obtained by differential migration of solutes in an electrical field. In CE, electrophoresis is performed in narrow-bore capillaries, which are usually filled with buffer. The use of high electrical fields results in short analysis times (a few minutes) and separation with high efficiency and resolution. The electro-osmotic flow (EOF) also allows the simultaneous analysis of all solutes, regardless of charge. The versatility of CE is partially derived from its numerous modes of operation. The separation mechanisms

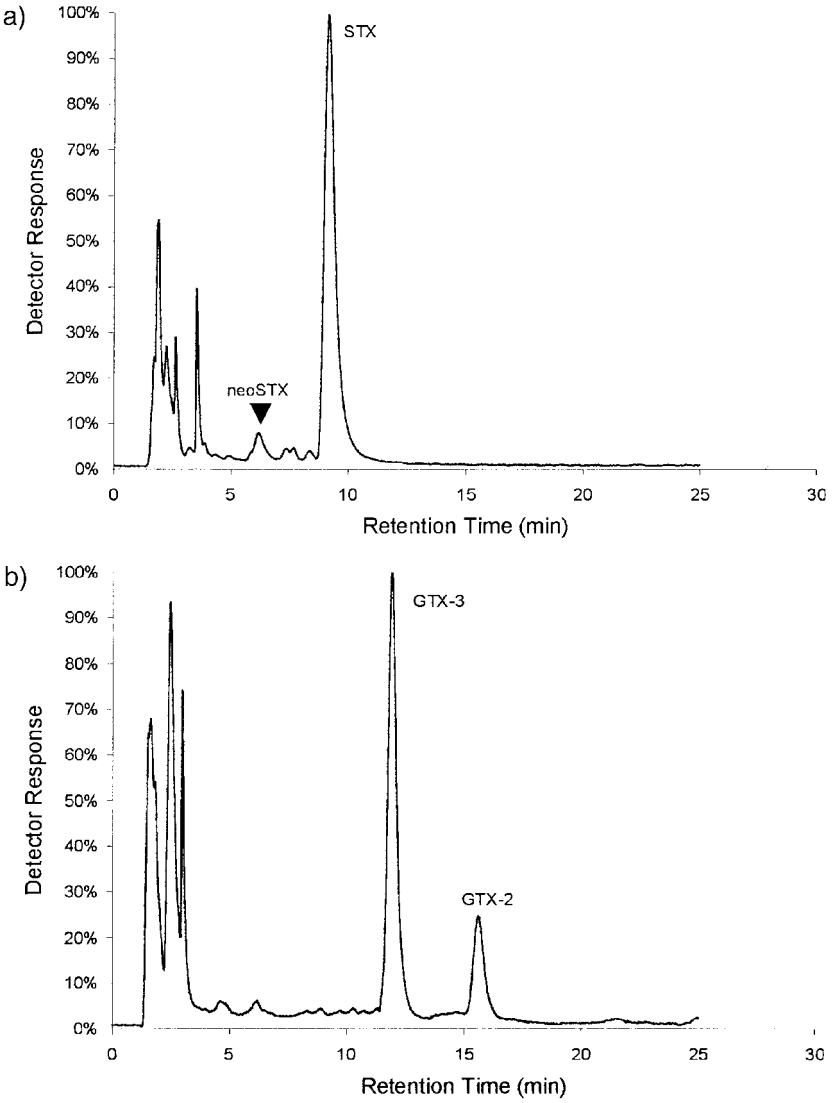


Figure 7.8
Representative HPLC trace of the carbonate PSP toxin components of the dinoflagellate strain *Alexandrium tamarense* PSP-4; Conditions: Inertsil C-18 column, 4.6 mm × 150 mm. Mobile phase: A, Eluent C and B, Eluent B (Oshima *et al*., 1995) at 0.8 ml min^{-1}; ECOS voltage 800 mV. Detector: 330 nm excitation; 390 nm emission.
Source: Boyer and Goddard (1999).

of each mode are different and thus can offer complementary information. Capillary zone electrophoresis (CZE) is fundamentally the simplest form of CE. In this procedure, the capillary is filled with buffer and separation occurs because solutes migrate

in discrete zones and at different velocities. In CZE, selectivity can most readily be altered through changes in running buffer pH or by use of buffer additives. In capillary isotachophoresis (CITP), a combination of two buffer systems is used to create a state in which the separated zones all move with the same velocity. The zones remain sandwiched between so-called leading and terminating electrolytes. In a single CITP experiment, either cations or anions can be analysed (Heiger, 1992). The application of on-column sample pre-concentration with CITP and discontinuous buffer systems prior to CZE has been investigated for the analysis of PSP toxins (Locke and Thibault, 1994). Separation by CITP/CZE of a mixture of eight PSP toxin standards is presented in Fig. 7.13.

Buzy *et al*. (1994) demonstrated the application of such a pre-concentration ('stacking') procedure prior to CZE for the analysis of decarbamoyl toxins. The separation conditions developed were found to be entirely compatible with electrospray MS. This permitted the analysis of PSP toxins and their decarbamoyl derivatives in crude enzyme digests. The products released during the enzymatic digestion were identified using CE combined with tandem MS. However, CE separations demand highly purified extracts to obtain reproducible separation. Additionally, given the extremely small volumes injected, the detection limit of this method is approximately an order of magnitude greater than for HPLC with fluorescence or MS detection.

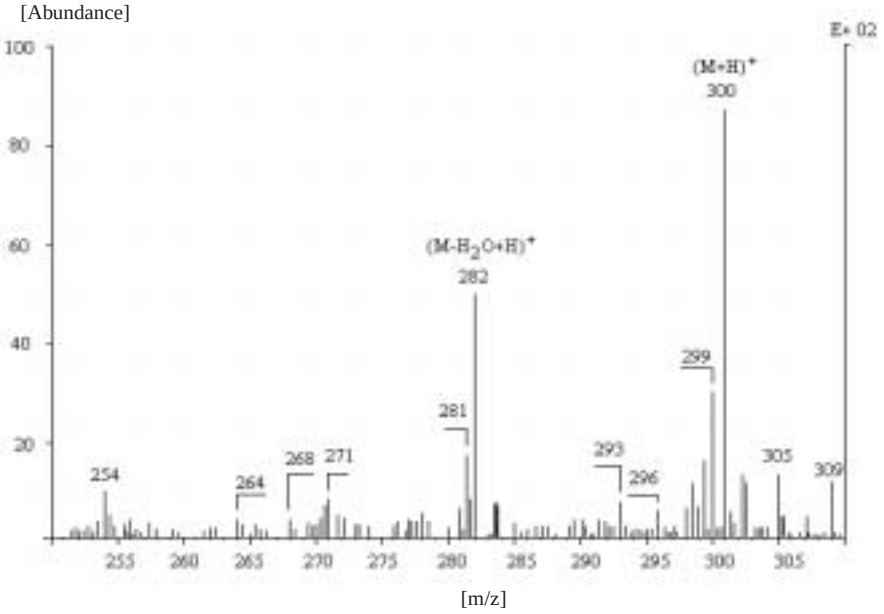


Figure 7.9
HPLC-Atmospheric Pressure Ion Source/Electrospray Ionization (API/ESI) mass spectrum of STX.
Source: Kirschbaum *et al*. (1995).

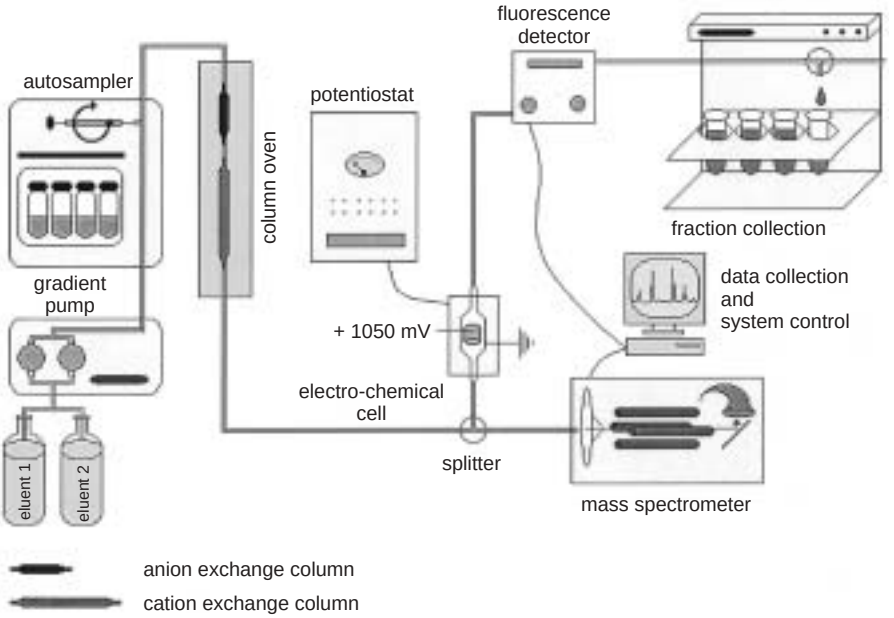


Figure 7.10
Automated HPLC system with ion-exchange columns, electrochemical cell with fluorescence detector and mass spectrometer (fraction collection as additional option).
Source: Jaime *et al*. (2000).

7.3 Conclusions

The more accurate the determination of the concentrations of the individual PSP toxins, the more accurate will be the calculation of total PSP toxicity. This follows from the fact that the total PSP toxicity is obtained by summing the toxicities of the individual toxin components, calculated from their concentrations and specific molar toxicities [in mouse units (MU) or STX equivalents]. Complete separation of PSP toxin components, the unambiguous assignment of peaks in the chromatograms, and the availability of suitable standards and reference materials, are prerequisites for accurate quantification. If these prerequisites are achieved, the results of chemical analysis can be compared with values determined by the mouse bioassay or other biological methods. Although the mouse bioassay is still obligatory in most countries for the quality control of PSP toxins in seafood, chemical analytical methods have much to contribute in reducing the dependence on the mouse bioassay for the determination of PSP toxins in contaminated seafood. Such analytical methods will continue to provide an essential means of toxin determination for research, regulatory purposes and confirmatory analysis.

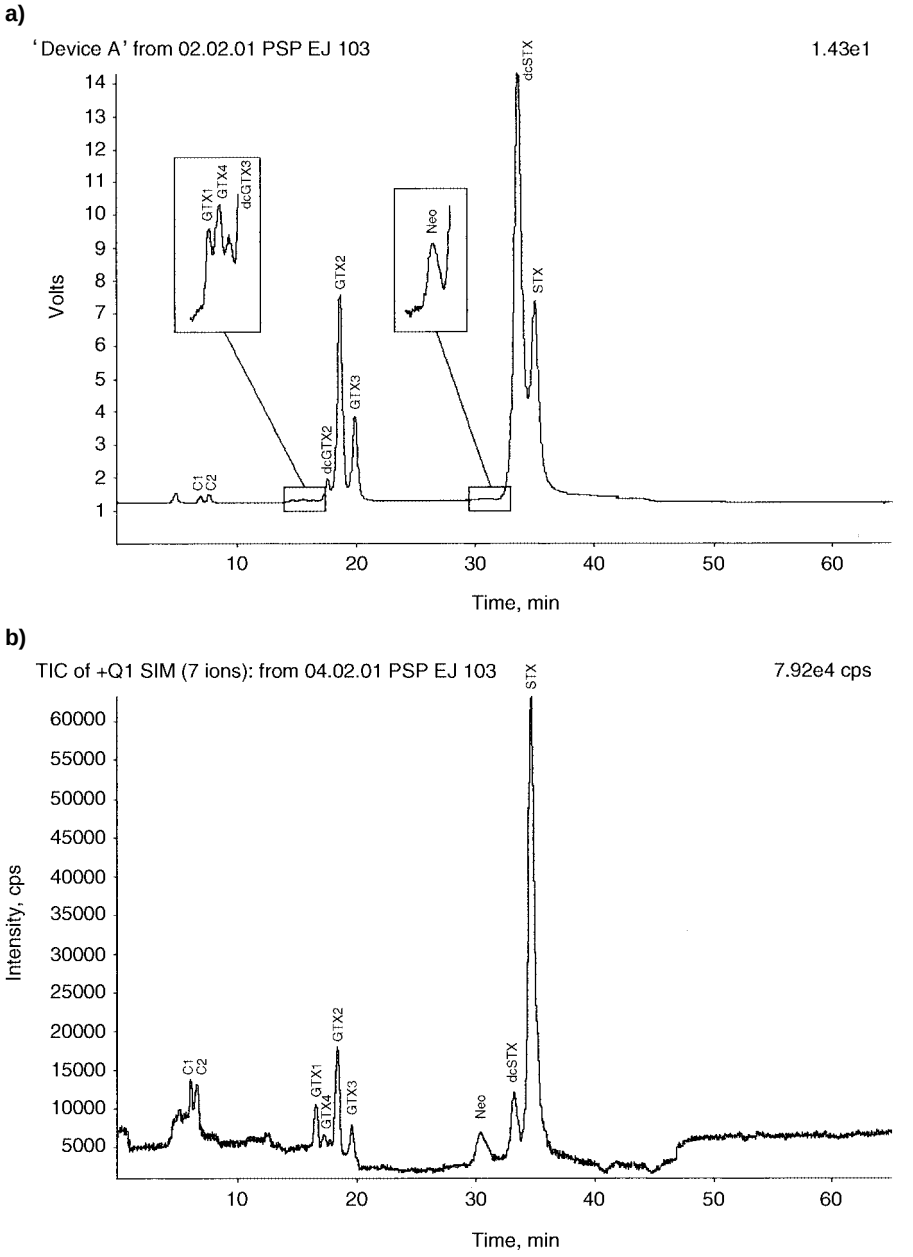


Figure 7.11
HPLC/FLD/MS determination of PSP toxins: C1–C2 (3.5 ng each), GTX1 (17.2 ng), GTX4 (8.9 ng), dcGTX3, dcGTX2, GTX2 (28.7 ng), GTX3 (10.4 ng), NEO (34.8 ng), dcSTX (25.0 ng), STX (39.9 ng). A, fluorescence chromatogram; B, TIC chromatogram.
Source: Jaime *et al.* (2001).

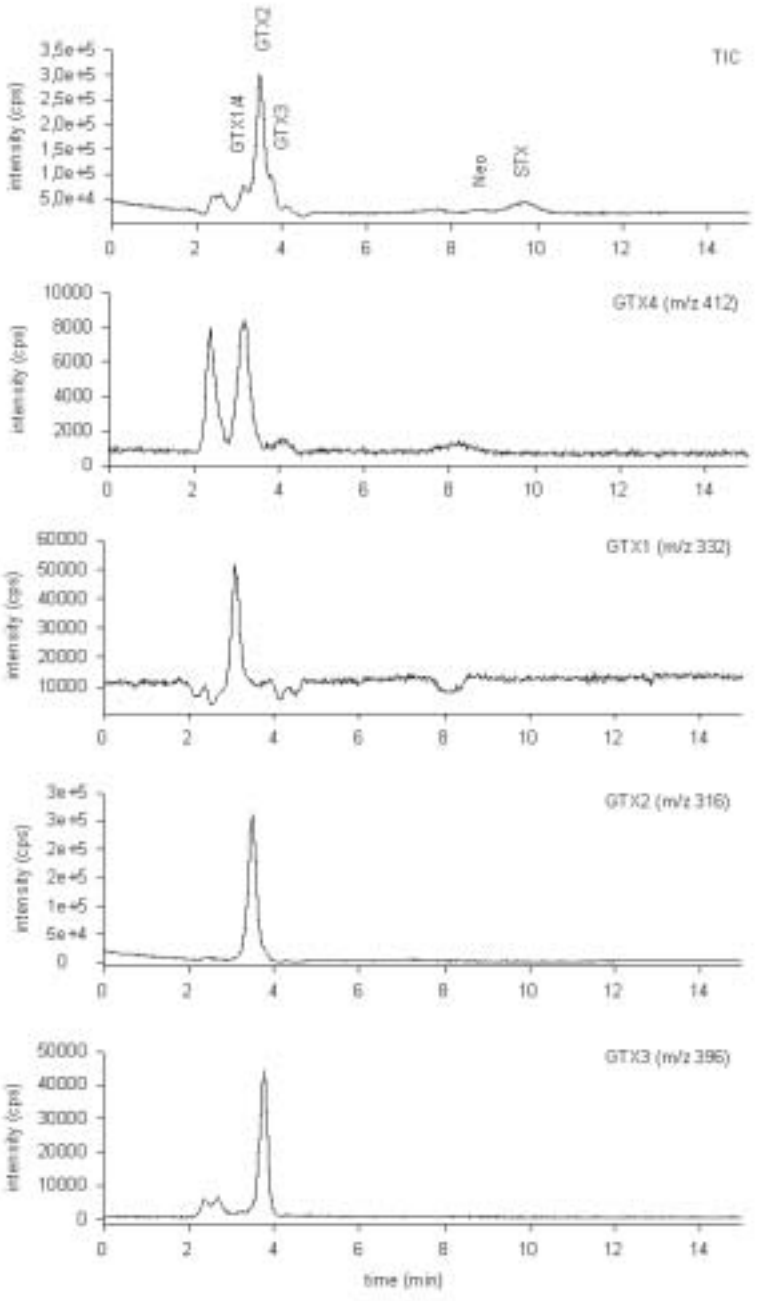


Figure 7.12
Ion-exchange HPLC/MS (Sim mode) determination of PSP toxins in mussels (*Mytilus chilensis*).
Source: Jaime *et al*. (2001).

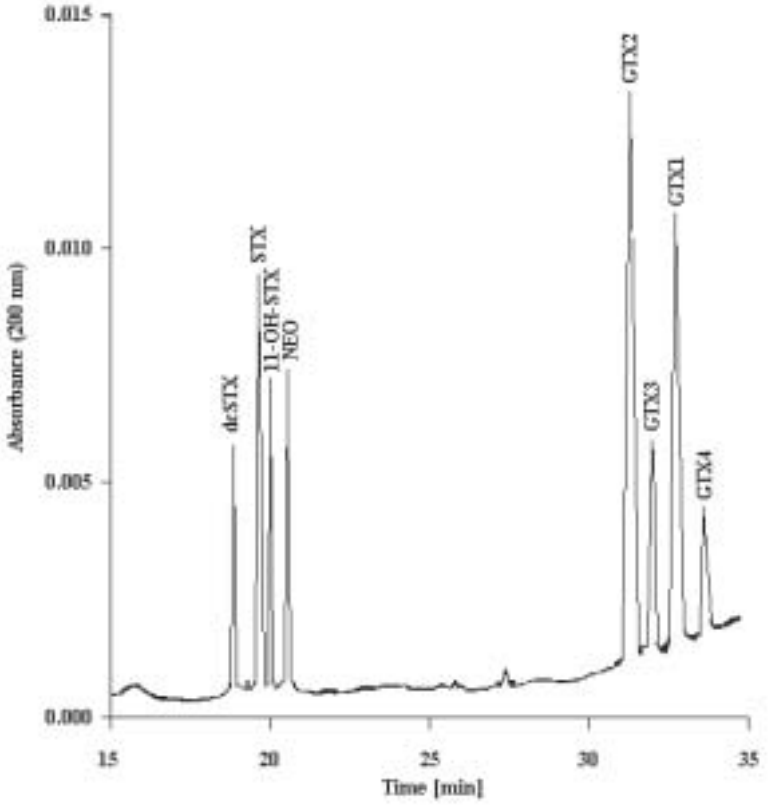


Figure 7.13
CITP/CEZ/UV analysis of a mixture of eight PSP toxin standards. Injection of 2.2 µl of a solution containing 500 ng ml^{-1} GTX1 and GTX2 and 200 ng ml^{-1} for all other PSP toxins.
Source: Locke and Thibault (1994).

APPENDIX 7.1

Sample preparation

Prior to the application of a chemical analytical method, PSP toxins must be quantitatively extracted from the sample matrix. The standard method for the mouse bioassay, using 0.1 N hydrochloric acid as the extraction solvent, was endorsed by the Association of Official Analytical Chemists (Hollingworth and Wekell, 1990) (see also Chapters 12 and 13 for more details and alternative protocols). The sample and the extraction solvent are heated for 5 min at 100°C. A fraction (non-quantitative) of the N-sulfo-carbamoyl toxins are converted into their respective carbamate analogues, i.e. B1, B2 and C1–C4 toxins are substantially, but not completely, converted in this procedure. Nevertheless, these AOAC extracts are suited for both the mouse bioassay and other methods of PSP determination (Fig. 7.14).

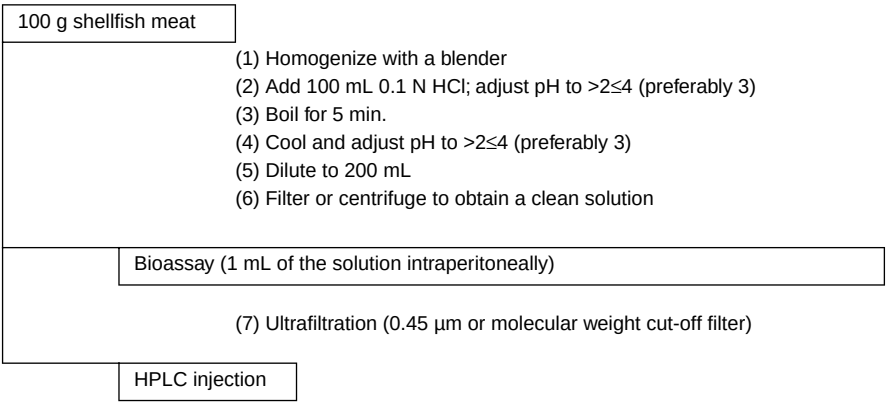


Figure 7.14
Sample preparation according to Association of Official Analytical Chemists (AOAC).
Source: Hollingworth and Wekell (1990).

Extraction with 0.1 N hydrochloric acid may be an inconvenience if the objective is to study the native toxin profile in biological material. However, the natural toxin composition can be analysed after extracting samples with acetic acid. Fresh sample material (1.0 g) is mixed with acetic acid (0.03 N, 3.0 ml) using a tissue homogenizer. The mixture is centrifuged for 10 min (2,980 × g) and passed through a 0.45 µm nylon filter. A second sample (1.0 g) is extracted with hydrochloric acid (0.2 N, 3.0 ml), with mixing, centrifugation, and filtration as described. Determination of N-sulfo-carbamoyl toxins is possible by calculating the peak height/area increases for the carbamoyl toxins formed after heating the HCl extract, following injection of the different extracts into the chromatograph (Hummert *et al.*, 1997).

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8

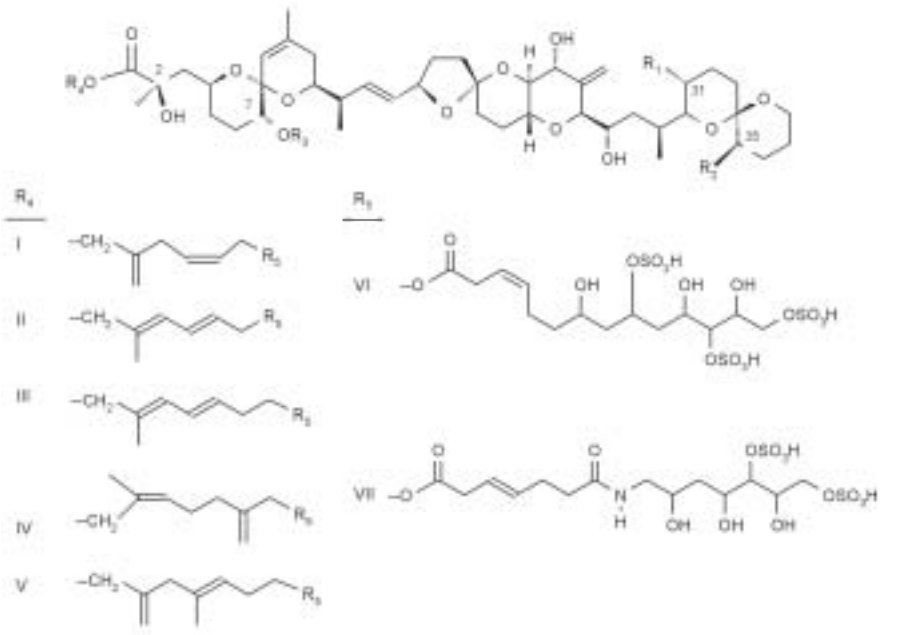
Chemical methods for lipophilic shellfish toxins

M. A. Quilliam

8.1 Introduction

The chemical properties and practical chemical analytical methods for lipophilic shellfish toxins are presented in this chapter. All these toxins are relatively non-polar, with molecular weights greater than 500, and are easily extracted into organic solvents such as methanol and chloroform. Most are polyether compounds, but they have distinctive chemical structures with widely varying functional groups and different toxicological and chemical characteristics. They are grouped here because of the potential for using a common sample extraction procedure. The ciguatera fish poisoning (CFP) toxins, ciguatoxin, maitotoxin and palytoxin, although lipophilic in nature, are not associated with shellfish poisoning (see Chapter 10).

Shellfish toxins have traditionally been categorized and regulated according to the poisoning syndrome that they induce in people or animals (see Chapters 13, 24 and 26). For example, okadaic acid (OA) and the dinophysistoxins (DTX) (see Fig. 8.1) have been classed as diarrhetic shellfish poisoning (DSP) toxins due to symptoms of diarrhoea (Yasumoto *et al*., 1985). Unfortunately, when pectentoxins (PTX) and yessotoxins (YTX) were discovered and before their toxicity was understood, they were placed into the "DSP" category because they often co-occur with OA and DTX and are detected by the mouse bioassay procedure first used to detect DSP toxins in lipophilic extracts. With the continuing discovery of an array of new lipophilic toxins such as azaspiracids, gymnodimines and spirolides, it has become clear that a better approach would be to categorize toxins strictly according to their chemical class rather than their toxic symptoms. This in turn would allow seafood safety to be regulated according to allowable levels of specific toxins rather than the outcome of a specific assay. This is the way in which all other environmental contaminants, such as mycotoxins and pesticides, are controlled. Analysts in regulatory laboratories would be allowed to use their choice of monitoring technology, provided that they have been properly validated. The choice could include a range of methods from rapid assay methods (see Chapter 12), such as immunoassays and receptor assays that can screen for specific toxin groups, to chemical methods that can provide detailed information on specific toxins. In fact, classification according to chemical groups is a prerequisite to the development of most assay and chemical analysis methods, the live animal bioassay being the exception. In this chapter, therefore, the toxins are discussed according to their structural groups (e.g. okadaic acid group) and not their toxic symptoms (e.g. DSP).



	R1	R2	R3	R4	R5	EMW
OA	CH_3	H	H	H	--	804.5
DTX1	CH_3	CH_3	H	H	--	818.5
DTX2	H	CH_3	H	H	--	804.5
DTX3	(H or CH_3)		Acyl	H	--	
OA-D7a	CH_3	H	H	I	OH	914.6
OA-D7b	CH_3	H	H	II	OH	914.6
OA-D8	CH_3	H	H	III	OH	928.6
OA-D9a	CH_3	H	H	IV	OH	942.6
OA-D9b	CH_3	H	H	V	OH	942.6
DTX4	CH_3	H	H	III	VI	1472.6
DTX5a	CH_3	H	H	II	VII	1392.6
DTX5b	CH_3	H	H	III	VII	1406.6

Figure 8.1
Structures of okadaic acid (OA) and dinophysistoxins (DTX). The diol ester derivatives of OA are designated by an OA-D#x code, where # is the number of carbons in the diol chain and x is a specific isomer.
EMW = exact molecular weight.

Methods for the determination of toxins may be divided into assay and chemical analytical methods. In assay methods, the measured signal is either a specific response to a single toxin structure or an integration of responses to several structures in a group (see Chapters 12 and 13). In order to use the assay result for evaluating seafood safety, it is most useful if an integrated response correlates with overall

toxicity. In a chemical analytical method, signals corresponding to individual toxin structures are measured. Most analytical methods are based on separation methods and allow the determination of several toxins in one analysis. Calculations of individual toxin concentrations require accurate standards to calibrate the responses and evaluation of seafood safety further requires specific toxicity factors. The most common analytical method for toxins is high-performance liquid chromatography (HPLC). Methods based on combined chromatographic and spectroscopic techniques, such as liquid chromatography-mass spectrometry (LC-MS), provide the greatest information and are unsurpassed for chemical and biochemical studies, for the unambiguous confirmation of known toxins, and for the identification of new toxins and metabolites.

There are many challenges to overcome before it is possible to fully implement chemical methods in comprehensive monitoring programmes and research studies. In particular, four critical developments are required before complete success can be claimed.

First, all members of a toxin class must be well characterized in terms of structure. We have accumulated a great deal of information on the structures of toxins that can contaminate shellfish, but each year the situation becomes more complex with the discovery of new toxin analogues and even new toxin classes. In addition, toxins produced by a particular microalgal species may be transformed in shellfish to metabolites, some toxic and others non-toxic. This is certainly a complicating factor in the development of methods and indicates a need to continue research on the identification of new toxins.

Second, accurate and readily available calibration solutions are required for each of the various toxins. This has always been a major stumbling block in shellfish toxin research and monitoring and much more effort has to be put into the development of reference materials and standards. The task is continually made more difficult by the growing list of toxins.

Third, the specific toxicity of individual toxins must be determined to allow calculations of overall toxic potential of samples. Risk assessment studies also need to be conducted to establish allowable levels of toxins in seafood.

Fourth, robust methods must be developed and validated for various shellfish tissues. They must provide adequate detection limits (low ng g^{-1} levels) and accurate quantitation of all individual toxin analogues within a toxin class. Many specific analytical methods have already been developed for individual toxins or toxin groups and the most useful ones are considered here. Unfortunately, because co-occurrence of toxin classes is a frequent problem, many laboratories must implement a multitude of specific procedures in order to analyse for all toxins comprehensively. A significant step forward would be the development of extraction and analysis methods that could determine multiple toxin classes with one procedure. In this regard, methods based on LC-MS appear to be the most promising.

8.2 Chemistry

8.2.1 Okadaic acid group

Okadaic acid (OA) and its analogues, DTX1 and DTX2, are the principal toxins responsible for incidents of diarrhetic shellfish poisoning (DSP) (Murata *et al*., 1982; Yasumoto *et al*., 1985; Hu *et al*., 1992a). Produced by *Dinophysis* and

Prorocentrum species, they are long chain compounds containing transfused or spiro-linked cyclic polyether rings with an α-hydroxy carboxyl function (see Fig. 8.1), differing only in the number of methyl groups at C31 and C35. Several isomers of OA and DTX have also been detected in shellfish and plankton (Quilliam, 1995; James *et al*., 1997; Draisci *et al*., 1998b) but their exact structures remain unsolved due to limited isolated quantities.

Okadaic acid and its analogues are very soluble in organic solvents such as methanol, acetone, chloroform, dichloromethane and dimethylsulfoxide. No significant chromophore is present, although there is some ultraviolet absorbance below 220 nm. The stability of OA in solution has been investigated during the preparation of certified calibration solutions (Quilliam, 1997). The best solvents were found to be methanol and acetonitrile, although OA has much lower solubility in the latter. Solutions of OA in some solvents, such as dimethylformamide and chloroform, can be unstable even at freezer temperatures. Dried residues of the pure compound on glass surfaces were found to decompose quite rapidly, although this does not appear to be as serious for crude extracts of plankton and shellfish, possibly due to a protective effect of the sample matrix. Okadaic acid is slightly sensitive to light, oxygen and heat. Therefore, for long-term storage of calibration solutions, it is recommended that the solution be degassed and stored under argon in amber-glass vials at -80°C. For short-term storage (less than three months) solutions may be stored in a freezer (-12°C or lower).

Several naturally occurring derivatives of OA and its analogues have been observed in plankton and shellfish. The term DTX3 was originally coined to describe a group of compounds in which saturated or unsaturated C_{14}–C_{18} fatty acyl groups are attached through the 7-hydroxy function of DTX1 (Yasumoto *et al*., 1985). Subsequently, it was shown that any of the parent toxins, OA, DTX1 or DTX2, can be acylated (Marr *et al*., 1992) (Fig. 8.1). These compounds are metabolic products in the shellfish and not *de novo* products of toxin-producing plankton (Suzuki *et al*., 1999).

Studies on cultures of *Prorocentrum lima* and *P. maculosum* have revealed a fascinating array of OA analogues in which the carboxyl group of OA is conjugated to several different unsaturated C_7 to C_9 diols to form allylic 'diol esters' (Fig. 8.1) (Yasumoto *et al*., 1987; Hu *et al*., 1992*b*). These same organisms also produce some water-soluble derivatives of OA, in which the diol esters are further conjugated to a polar side chain. Dinophysistoxin-4 (DTX4) (Fig. 8.1) is one of these in which OA is coupled via a C_9 diol group to a C_{14} aliphatic chain with three hydroxyl and three sulfate groups (Hu *et al*., 1995c). Dinophysistoxin-5 (DTX5a and DTX5b) (Hu *et al*., 1995*b*) are two similar compounds but these possess an amide function in the polar side chain. The situation appears to be even more complex than that shown in Fig. 8.1 because a wide variety of DTX4 and DTX5 analogues have been detected in various *Prorocentrum* species (Quilliam *et al*., 1996). LC-MS analyses indicated that these compounds were based on either OA or DTX1, coupled via different diol linkages to aliphatic chains modified with varying numbers of sulfates and alcohol functions. Although these compounds do not appear to be active as phosphatase inhibitors, they can be hydrolysed chemically or enzymatically to yield the active parent DSP toxins. Quilliam and co-workers (1996) showed that the diol esters are, in fact, artefacts of sample preparation due to a partial hydrolysis of DTX4. Both ester linkages in DTX4 are easily hydrolysed by an esterase that is present in the plankton and is released when the cells rupture due to rough handling or a freeze/

thaw cycle (Quilliam *et al*., 1996). Conversion of DTX4 to the diol ester proceeds very rapidly (in less than a minute at room temperature) while the conversion of the diol ester to OA proceeds much more slowly (over several hours). Isolation and analysis of DTX4 and DTX5 is facilitated by destroying esterases in plankton by boiling immediately after isolation of the cells and prior to extraction (Quilliam *et al*., 1996). Dinophysistoxin-4 and -5 have not been observed in shellfish, probably because of their lability to esterases in the plankton or shellfish tissues (Quilliam, unpublished data).

Dinophysistoxin-3 (DTX3) and the diol esters are considerably more lipophilic than the parent compounds and can therefore be partially lost to hexane in some of the published liquid-liquid partitioning procedures. If an aqueous methanol extract of plankton is partitioned against hexane, diethyl ether and 1-butanol, OA and its lipid soluble derivatives are found mainly in the ether layer, while DTX4 and DTX5 transfer to the butanol phase.

All the various ester derivatives (DTX3, diol esters, DTX4 and DTX5) can be hydrolysed to the parent toxins quantitatively by treatment with 0.25 M NaOH in methanol-water (8:2, v/v) at 35°C for 40 min (Vale and Sampayo, 1999). Such a procedure can be used to simplify analysis (see Appendix 8.2).

8.2.2 Pectenotoxin group

The pectenotoxin (PTX) group, named after the genus of scallop from which they were first isolated, *Patinopecten yessoensis* (Yasumoto *et al*., 1985), is comprised of several cyclic polyether macrolides (Fig. 8.2). PTX2 is the main toxin produced by *Dinophysis* species, such as *D. fortii*. When accumulated in shellfish, the methyl group at C43 is oxidized to the alcohol (PTX1), aldehyde (PTX3), and carboxylate (PTX6) forms. The spiroketal ring system in rings A and B can also undergo rearrangement and/or epimerization under acidic conditions to produce PTX4 and PTX7 through PTX9 (Sasaki *et al*., 1998). The lactone ring in PTX2 may be opened to yield pectenotoxin seco acid (PTX2sa), epimerization of which yields 7-*epi*-PTX2sa (Daiguji *et al*., 1998*a*). This transformation has been shown to occur enzymatically in shellfish (Suzuki *et al*., 2001) and even in plankton that has been taken through a freeze/thaw cycle (Quilliam, unpublished data).

All the pectenotoxins absorb in the UV between 235 nm and 239 nm. Detailed stability studies have not been performed, but it is known that rearrangements can occur under acidic conditions. These compounds are easily destroyed under strong basic conditions such as those used to hydrolyse acyl esters of the OA group.

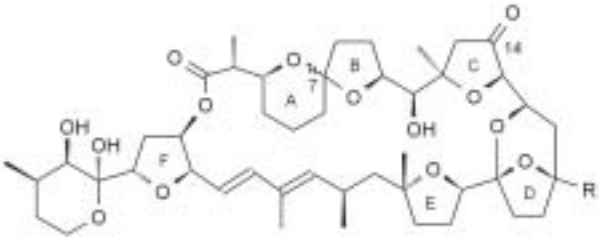
8.2.3 Yessotoxin group

Yessotoxin (YTX) is another type of toxin first isolated from the Japanese scallop *Patinopecten yessoensis* (Fig. 8.3) (Murata *et al*., 1987). This ladder-shaped polycyclic ether compound is produced by the dinoflagellate *Protoceratium reticulatum* (Satake *et al*., 1997*a*). A number of YTX analogues have also been identified and are given in Fig. 8.3. Homoyessotoxin (homo-YTX) is produced by another planktonic dinoflagellate *Lingulodinium polyedrum* (Tubaro *et al*., 1998). Both toxins are oxidized to 45-hydroxy derivatives in shellfish (Satake *et al*, 1997*b*). Other compounds include a 1-desulfo derivative of YTX (Daiguji *et al*., 1998*b*), carboxy derivatives of YTX and homo-YTX (Ciminiello *et al*., 2000*a*, 2000*b*), and adriatoxin (ATX) (Ciminiello *et al*., 1998).

All the yessotoxins, except the carboxy derivatives and ATX, possess a conjugated diene, which can provide UV absorbance at 225–230 nm. The stability of yessotoxins has not been investigated in detail.

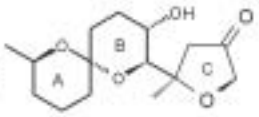
8.2.4 Azaspiracid group

The major toxin in this new group of toxins is named azaspiracid (AZA) because of its unusual azaspiro ring assemblies and the presence of a carboxyl function (Fig. 8.4) (Satake *et al*., 1998). Two homologues, 8-methylazaspiracid (AZA2) and 22-desmethylazaspiracid (AZA3), and two oxidized analogues, 3-hydroxy-22-desmethylazaspiracid (AZA4) and 23-hydroxy-desmethylazaspiracid (AZA5), have

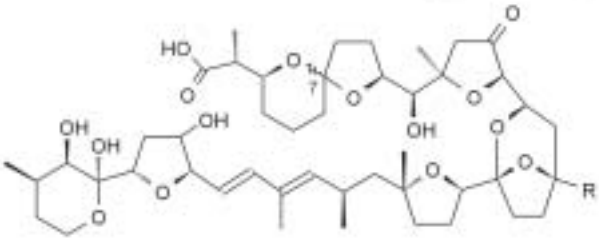


	R	C7	EMW
PTX2	CH_3	R	858.5
PTX1	CH_2OH	R	874.5
PTX4	CH_2OH	S	874.5
PTX3	CHO	R	872.5
PTX5 (1)	CH_2OH	R	876.5
PTX6	COOH	R	888.5
PTX7	COOH	S	888.5

(1) carbonyl at C14 reduced to hydroxyl



PTX8	CH_2OH	S	874.5
PTX9	COOH	S	888.5



PTX2sa	CH_3	R	876.5
7-epi-PTX2sa	CH_3	S	876.5

Figure 8.2
Structures of the known pectenotoxins (PTX) and pectenotoxin seco acids (PTX2sa).

been reported recently (Ofuji *et al*., 1999*a*, 2001). The carboxyl and amine functions in these azaspiracids appear to form an intramolecular ion-pair. This can result in an overall lower apparent polarity for the molecule and a lower reactivity of both functions, thus making it difficult to prepare derivatives for fluorescence detection in HPLC. Azaspiracid appears to be unstable in some solvents such as chloroform, under slightly alkaline conditions, and during chromatography on silica-based supports (James *et al*., 2000).

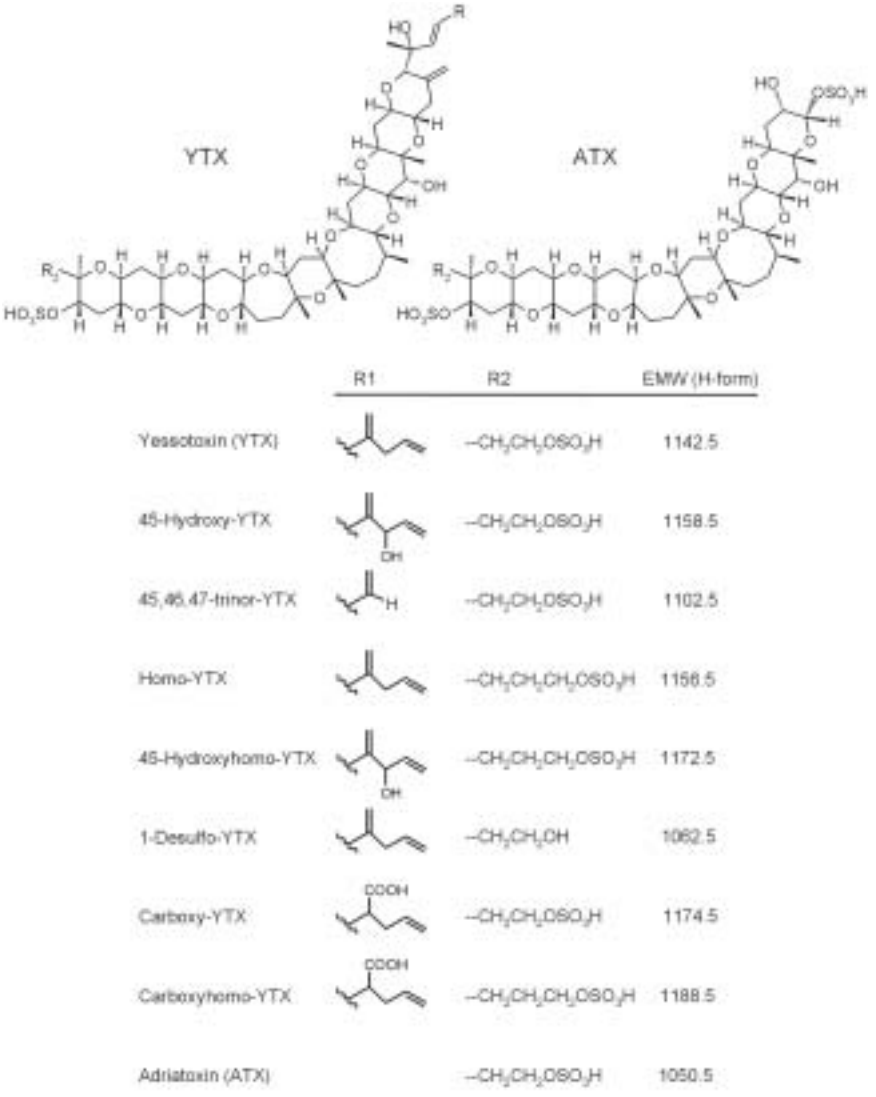
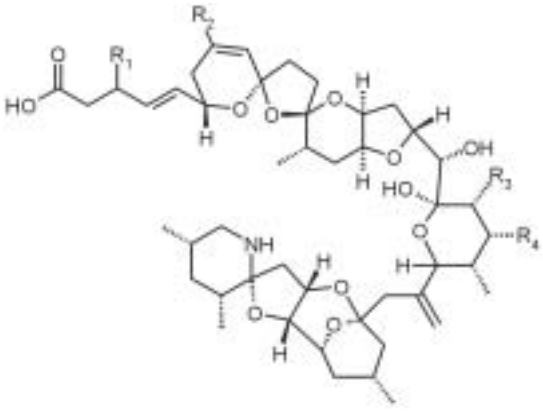


Figure 8.3
Structures of yessotoxins (YTX) and adriatoxin (ATX).
The stereochemistry in ATX is relative, not absolute.



Azaspiracid	R1	R2	R3	R4	EMW
AZA	H	H	CH_3	H	841.5
AZA2	H	CH_3	CH_3	H	855.5
AZA3	H	H	H	H	827.5
AZA4	OH	H	H	H	843.5
AZA5	H	H	H	OH	843.5

Figure 8.4
Structures of azaspiracids.

8.2.5 Cyclic imine toxins

In recent years, a variety of toxins with a cyclic imine function and macrocyclic structure have been discovered through their action as 'fast acting' toxins in the mouse bioassay. They include gymnodimines (Fig. 8.5) (Seki *et al*., 1995; Miles *et al*., 2000), spirolides (Fig. 8.6) (Hu *et al*., 1995*a*, 1996, 2001), and pinnatoxins and pteriatoxins (Fig. 8.7) (Uemura *et al*., 1995; Chou *et al*., 1996; Takada *et al*., 2001*a*, 2001*b*). Only the pinnatoxins have been implicated in incidents of human illness, so it remains to be determined if these compounds are of serious concern for seafood safety. Compounds such as the shellfish metabolites, spirolides E and F (Fig. 8.6) in which the cyclic imines of spirolides A and B have been opened to a keto amine, are not toxic (Hu *et al*., 1996).

Gymnodimines and spirolides are stable in methanol or acidic aqueous methanol solutions but have been found to be quite unstable in aqueous solutions at a pH greater than 5 (Miles and Quilliam, unpublished data). The instability of these toxins at physiological pH could have serious implications in toxicological studies.

8.2.6 Brevetoxin group

Brevetoxins (BTX), produced by the dinoflagellate *Karenia brevis* (formerly known as *Gymnodinium brevis* and *Ptychodiscus brevis*), are ladder-shaped polycyclic ether

compounds based on two different structural backbones (type A or type B, Fig. 8.8) (Baden, 1989; Baden and Adams, 2000). A common feature of these neurotoxins, besides the relatively rigid ring systems, is a lactone ring that is required for activity. Recently, several complicated metabolites of brevetoxins have been identified in shellfish (Fig. 8.9) (Ishida *et al*., 1995; Morohashi *et al*., 1995, 1999; Murata *et al*., 1998). The toxicological significance of these metabolites is still under investigation.

The stability of the brevetoxins stored at reduced temperatures in solution or in a dry state has been reported to be good (Baden, 1989). Decomposition occurs in aqueous solutions below pH 2 or above pH 10, the latter through saponification of the lactone ring. Brevetoxins with an aldehyde function in the terminal side chain are decomposed under acidic conditions (Hua and Cole, 1999).

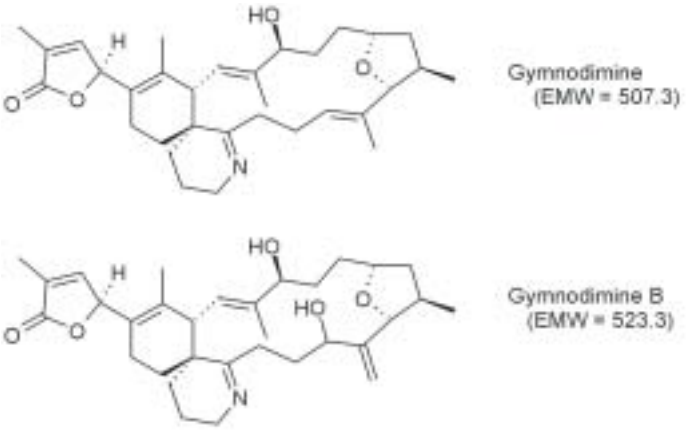


Figure 8.5
Structures of gymnodimine and gymnodimine B.

8.3 Chemical analysis

8.3.1 Extraction and clean-up methods

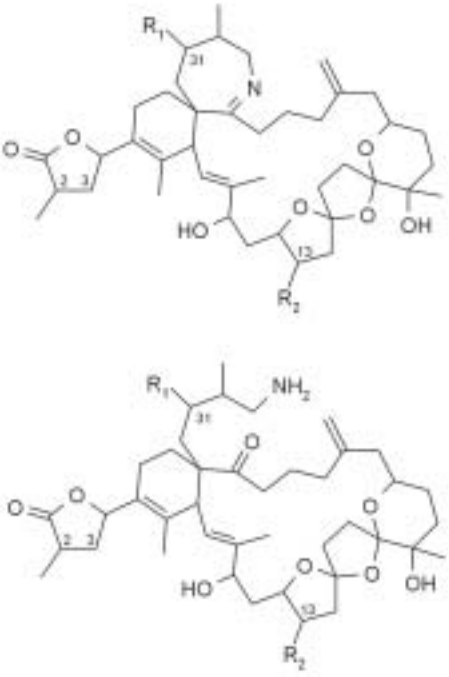
All the lipophilic toxins can be extracted from plankton and shellfish tissues with organic solvents such as acetone, methanol and acetonitrile. Acetone has been used in many preparative and bioassay procedures because of the ease with which the solvent may be evaporated. Unfortunately, acetone extracts are very complex due to the presence of fats and other non-polar lipids. Evaporating the acetone and then partitioning the residue between petroleum ether or hexane and aqueous methanol can greatly reduce the lipid content of the extract. Such procedures are very labour-intensive and consume large quantities of flammable solvents.

A better extraction solvent for chemical analysis is 80% methanol in water, which greatly reduces the amount of lipids extracted. Good recovery of most toxins can be attained with a single-step homogenization of 8 ml of solvent with 2 g tissue. Partitioning against hexane can still be used to remove lipids from the crude methanol extract, unless one is concerned with loss of low polarity toxins such as DTX3

(Fernández *et al*., 1996). After addition of more water to reduce the methanol to approximately 50%, toxins can be partitioned into chloroform or dichloromethane, which is then evaporated to dryness to yield an oily residue that can usually be redissolved in 0.5–1 ml of methanol. A detailed procedure that has been fully validated is provided in Appendix 8.2 for the analysis of the okadaic acid group (Quilliam, 1995). Ethyl acetate is a promising alternative to the use of chlorinated solvents (Gonzalez and Botana, 2000), but requires a drying step (sodium sulfate) to remove dissolved water prior to evaporation.

It should be noted that in many of the published procedures for the analysis of shellfish, only digestive glands are extracted as most lipophilic toxins accumulate primarily in these organs. This provides an amplification factor of 5 to 10 that can facilitate better method detection limits. Unfortunately, as some toxins such as gymnodimine and azaspiracids may be distributed throughout the tissues of the animal, one has to be careful with such a practice for multi-toxin screening.

The use of more sensitive and selective analytical methods, such as LC-MS, can sometimes permit a simpler extraction and can avoid dissecting-out digestive glands. In addition, as indicated in Section 8.3.4, the LC-MS method is suitable for the simultaneous determination of multiple toxins. For this to succeed, it is important to find a universal extraction solvent and clean-up scheme that can give good recovery for all toxins. Appendix 8.4 presents a procedure for the analysis of a wide range of

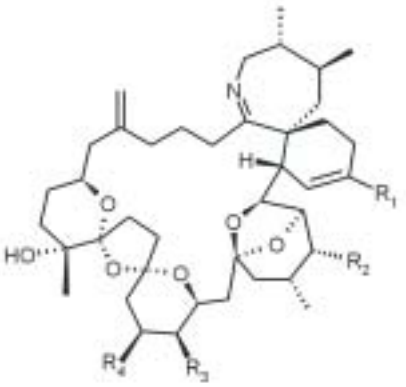


Spirolide	R1	R2		EMW
A	H	CH_3	$\Delta^{2,3}$	691.5
13-desMe-C	CH_3	H	$\Delta^{2,3}$	691.5
B	H	CH_3		693.5
C	CH_3	CH_3	$\Delta^{2,3}$	705.5
D	CH_3	CH_3		707.5
E	H	CH_3	$\Delta^{2,3}$	709.5
F	H	CH_3		711.5

Figure 8.6
Structures of known spirolides. The $\Delta^{2,3}$ indicates that there is a double bond between carbons 2 and 3.

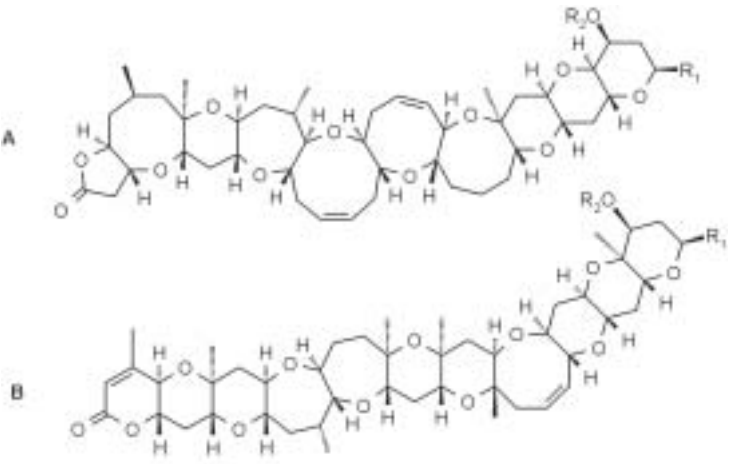
lipophilic toxins in whole edible tissues, using an aqueous methanol extraction and chloroform partitioning (without hexane). This is a provisional method that has been used successfully for the screening of samples for lipophilic shellfish toxins, but it has not yet been fully validated for quantitative analysis (Quilliam, unpublished data). In addition, more work needs to be done on sample clean-up procedures that would provide increased selectivity and a higher pre-concentration factor.

Further clean-up of extracts may be accomplished with the use of various types of column chromatography to isolate specific groups of toxins. Examples include the clean-up of brevetoxins using silica columns (Pierce *et al*., 1990) or a combination of alumina column and gel permeation chromatography (Shea, 1997). The clean-up of OA and analogues has been accomplished using gel permeation chromatography (Hummert *et al*., 2000). The problem with such methods is that they can be quite time-consuming and therefore do not lend themselves very well to routine analyses. An area where more research should be focused is the application of solid phase extraction (SPE) cartridges, which can be quite economical. A C18-silica SPE method for cleaning yessotoxins prior to derivatization and LC-FLD analysis is presented in Appendix 8.3.



		R1	R2	R3	R4	EMW
Pinnatoxin	A	COOH	OH	H	H	711.4
	B	$CH(NH_2)COOH$ *(S)*	OH	H	H	740.5
	C	$CH(NH_2)COOH$ *(R)*	OH	H	H	740.5
	D	$COCH_2CH_2COOH$	H	OH	CH_3	781.5
Pteriatoxin	A	$CH(OH)CH_2SCH_2CH(NH_2)COOH$	OH	H	H	830.5
	B,C	$CH(CH_2OH)SCH_2CH(NH_2)COOH$	OH	H	H	830.5

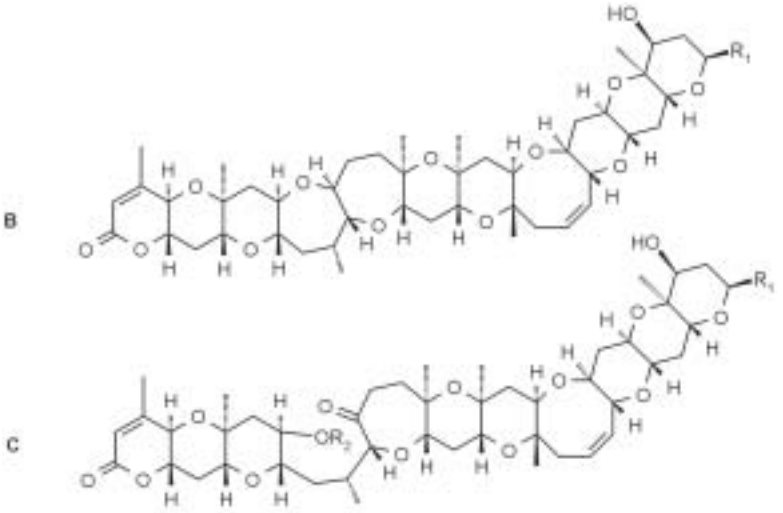
Figure 8.7
Structures of pinnatoxins and pteriatoxins.



	Backbone	R1	R2	EMW
BTX1	A		H	868.5
BTX7	A		H	868.5
BTX10	A		H	870.5
BTX2	B		H	894.5
BTX3	B		H	896.5
BTX9	B		H	898.5
BTX8	B		H	916.5
BTX6	B	27,28-epoxide + 	H	910.5
BTX5	B		-COCH$_3$	936.5

Figure 8.8
Structures of brevetoxins.

Immunoaffinity columns also hold great promise for cleaning up complex sample extracts. In this technique, an antibody to the toxin is bound to a sorbent in a column. This approach is quite common in the mycotoxin field, but has only been reported for the analysis of OA and analogues (Puech *et al*., 1999; Delaunay *et al*., 2000). One difficulty with applying this technique to the lipophilic toxins is the problem of non-specific adsorption on the packing material due to low solubility of the toxins in the primarily aqueous mobile phases that are typically used.



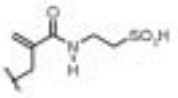
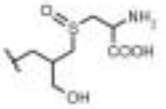
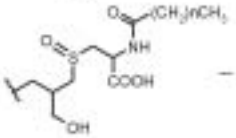
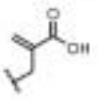
	Backbone	R1	R2	EMW
BTX-B1	B		–	1017.5
BTX-B2	B		–	1033.5
BTX-B4	B		–	n = 12 1243.7 n = 14 1271.7
BTX-B3	C		$-CO(CH_2)_nCH_3$	n = 12 1136.7 n = 14 1164.7

Figure 8.9
Structures of brevetoxins (continued).

A useful procedure for simplifying the analysis of the okadaic acid group of toxins is a base hydrolysis to convert acyl esters such as DTX3 to the parent toxins, OA, DTX1 or DTX2. A procedure for this is given in Appendix 8.2. It is important to bear in mind that other toxins, such as the pectenotoxins, can be destroyed by such treatment.

8.3.2 High-performance liquid chromatography

High-performance liquid chromatography (HPLC) is the most widely used instrumental analytical tool for shellfish toxins because it is well suited to the separation of a wide range of toxin structures, provides excellent quantitative precision, and is easily automated. A variety of detection systems are available, including UV absorbance (UVD), fluorescence (FLD), refractive index, electrochemical, evaporative light scattering, and mass spectrometry (MS). HPLC-UVD and HPLC-FLD methods are presented in this section. LC-MS techniques are discussed separately in Section 8.3.4.

HPLC-UVD has found very limited application to the analysis of lipophilic toxins. This method has neither the selectivity nor the sensitivity required for the determination of toxins at trace levels (ng g^{-1}). Organic extracts of shellfish tissues and plankton are extremely complex, even after liquid-liquid partitioning steps to remove fats and other lipids. Only a few of the toxins have UV-absorbing chromophores (e.g. the diene function in the PTX group) but many other co-extracted substances do absorb strongly in the UV range. Most of the lipophilic toxins have some absorbance below 210 nm but extinction coefficients are quite low. Operation at wavelengths below 210 nm places severe limitations on the HPLC operation. Only acetonitrile-water mobile phases with transparent modifiers such as trifluoroacetic acid can be used. Gradient elution is difficult because of severe baseline drifts. HPLC-UVD is useful for some research operations, such as the preparative isolation of toxins for structure studies or the examination of bulk plankton samples after an initial clean-up by column chromatography. Pierce and co-workers (1990) have analysed brevetoxins in plankton using reversed-phase HPLC and low-wavelength (215 nm) UV detection. The extracts were cleaned extensively using a double liquid-liquid partitioning procedure as well as silica-gel column chromatography. Linear response was achieved over the range 100–2,000 ng injected.

A more useful approach to HPLC analysis is to use chemical derivatization to introduce a UV-absorbing or fluorescent moiety into the molecule. This requires the presence of a reactive functional group in the analyte and a selective reagent that can react quantitatively under fairly mild conditions over a short period. A little-known key feature of a derivatization method is that it is possible, in principle, to use one compound as a calibrant for the analysis of another compound similar in structure. Given a quantitative extraction and conversion to the derivative and if the introduced fluorescent group is the only contributor to the analytical signal, then the molar response for all derivatized compounds should be the same. For example, one compound such as OA can be used to calibrate for determination of DTX1 and DTX2. Isocratic elution must be used because a change in solvent composition during a gradient could affect molar response.

It is not possible to provide a comprehensive review of all the various derivatization and LC-FLD methods that have been developed for lipophilic toxins. A few of the published methods are highlighted and detailed methods for the OA/DTX and YTX groups are presented.

The OA/DTX group of toxins is the most thoroughly studied because the free carboxyl functions allow the molecules to be tagged by a number of different derivatization reagents. Conjugation of the carboxyl group in the diol ester and DTX4/5 analogues prevents direct derivatization, so the ester bonds must be hydrolysed to release the parent toxins, as discussed in Section 8.3.1. Early work by Lee *et al.* (1987, 1989) established the suitability of 9-anthryldiazomethane (ADAM) for derivatization of OA and DTX1. The ADAM reagent is highly selective for carboxyl functions. This method has been used in many research laboratories but problems were encountered in the implementation of the method for routine monitoring. The low purity of the ADAM reagent (which results from its poor stability), and the presence of numerous other reactive co-extractives in shellfish tissues, necessitate a silica-column clean-up following the derivatization step. An extensive study of the ADAM procedure (Quilliam, 1995) has shown that it is necessary to pay particular attention to the silica clean-up following the derivatization step. The activities of both the silica gel and solvents must be carefully controlled. An internal standard, acetylokadaic acid, was also developed and shown to provide improvements in precision and accuracy by correcting for incomplete recovery in extraction, clean-up and derivatization steps and for volumetric errors and instrumental drift (Quilliam, 1995). A detailed procedure based on these developments is provided in Appendix 8.2. Fig. 8.10 shows typical HPLC-FLD analyses of ADAM-derivatized standards and some

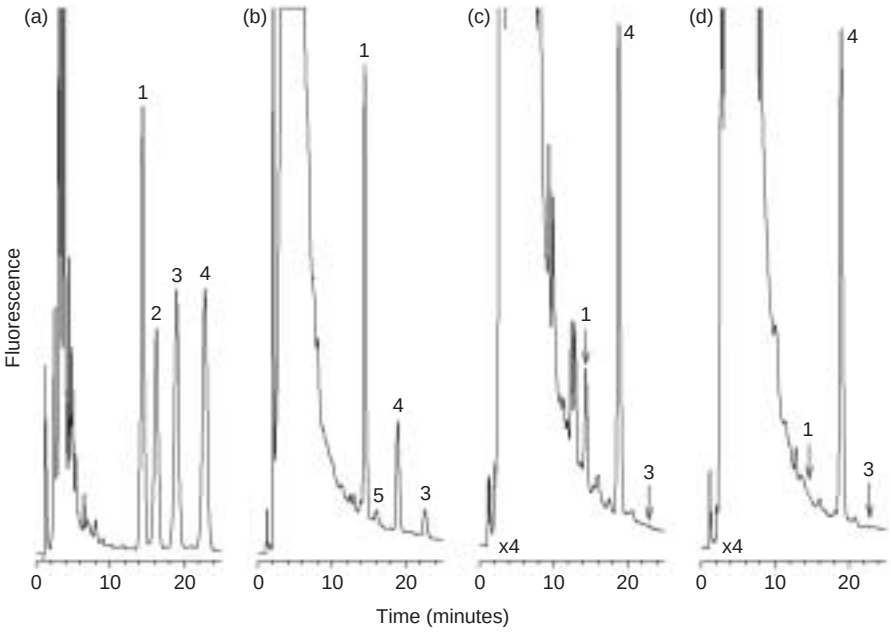


Figure 8.10
HPLC-FLD analysis of ADAM derivatives of: A, calibration standard mixture; B, MUS-2 certified mussel tissue reference material (11 mg kg^{-1} OA, 0.9 mg kg^{-1} DTX1); C, contaminated mussels (0.94 mg kg^{-1} OA); D, uncontaminated control mussels. Peak identities: 1, OA; 2, DTX2; 3, DTX1; 4, acetyl-OA (internal standard); 5, isomer of OA. Chromatographic conditions are given in Appendix 8.2.

mussel samples. The ADAM method is very sensitive for DSP toxins, being able to detect as little as 10 pg of the OA derivative injected on-column. The minimum detectable concentration in shellfish tissue, however, is limited not by detector sensitivity but by chemical background, which can vary considerably between samples. The practical quantitation limit is about 100 ng g^{-1} tissue. If digestive glands only are used in the analysis this limit translates to 10–20 ng g^{-1} for whole tissue for mussels. More recent studies have reported further improvements to the ADAM method. Quilliam *et al*. (1998) investigated the use of *in situ* production of ADAM from the more stable reagent, 9-anthraldehyde hydrazone. This procedure should be useful for those laboratories that have trouble acquiring and storing the ADAM reagent. Hummert *et al*. (1996) used automated column switching during the HPLC analysis to eliminate the need for silica SPE clean-up after derivatization. Finally, Ramstad *et al*. (2001*b*, 2001*c*) have made a thorough evaluation of HPLC methods and found the ADAM method to be the most reliable.

Other derivatization reagents have been investigated for the OA/DTX group but none have proven as selective in their reaction as the ADAM reagent. The reagent most studied next to ADAM is 1-bromoacetylpyrene (BAP) (Dickey *et al*., 1993; Kelly *et al*., 1996). The use of this reagent has recently been optimized and validated (Gonzalez *et al*., 1998; Gonzalez and Botana, 2000). Its main advantage over ADAM is its stability. Other reagent systems include 2-(anthracene-2,3-dicarboximido)ethyl-trifluoromethanesulfonate (AE-OTf) (Akasaka *et al*., 1996*b*), several coumarins (Marr *et al*., 1994; Ramstad *et al*., 2001*b*, 2001*c*), and luminarin-3 (James *et al*., 1998). The AE-OTf reagent has also been applied to the determination of DTX3 toxins (Akasaka *et al*., 1996*a*).

Yasumoto and Takizawa (1997) developed a selective derivatization procedure for the HPLC-FLD determination of the yessotoxin group of toxins. As most members of this group have a conjugated diene in the side-chain (adriatoxin being the exception), a dienophile reagent, (4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD), was used to convert them into fluorescent derivatives. Two chromatographic peaks are produced for each toxin due to the formation of two epimers in an approximately 2:1 ratio. An instrumental detection limit of 1 ng YTX was reported, suggesting a possible method detection limit of 100 ng g^{-1}. Details of this method are presented in Appendix 8.3. Ramstad *et al*. (2001*a*) performed a validation study which showed that this HPLC method gives repeatable results, superior to those achieved with mouse bioassay.

Methods for the pectenotoxin group have also been developed. ADAM derivatization has been used for PTX with a carboxyl function, PTX6, PTX7, PTX9 and the PTX2 seco acids (Yasumoto *et al*., 1989; Daiguji *et al*., 1998*a*; James *et al*., 1999). The compound BAP has also been used for acidic PTX (James *et al*., 1999). PTX1 and PTX4 can be derivatized with 1-anthroylnitrile, which reacts with primary hydroxyl functions under mild conditions (Yasumoto *et al*., 1989, 1995). The DMEQ-TAD reagent has also been used recently for the determination of PTX, which all possess a conjugated diene function (Sasaki *et al*., 1999).

8.3.3 Capillary electrophoresis

Capillary electrophoresis (CE) is a relatively new technique that offers some unique opportunities for the analysis of marine toxins. It is a relatively inexpensive method that provides fast, high-resolution separations. A key feature is that very little sample

is consumed, with injection volumes typically being 10–100 nl. This is particularly attractive for plankton research where sample size may be limited. For lipophilic toxins with a limited aqueous solubility, the preferred CE technique is micellar electrokinetic capillary chromatography (MECC), in which a surfactant is added to the running buffer. The surfactant helps to dissolve low polarity substances, while minimizing wall interactions and achieving separations of non-ionic compounds.

A very impressive demonstration of the technique is the analysis of brevetoxins by MECC with laser-induced fluorescence (LIF) detection (Shea, 1997). Brevetoxins with an alcohol group are derivatized with an acyl azide coumarin to form stable, highly fluorescent products. Brevetoxins with an aldehyde group are reduced to the alcohol with sodium borohydride prior to derivatization with coumarin. Detection with excitation at 354 nm (He-Cd laser) and emission at 410 nm gives an instrumental detection limit of 0.10 fg (10^{-16} g). Method detection limits for brevetoxins in fish tissues are approximately 4 pg g^{-1}. One limitation of the method is that it requires an extensive clean-up procedure based on a combination of alumina column and gel permeation chromatography.

The only other published application of CE to lipophilic toxins is an MECC-UVD method for OA (Bouaicha *et al*., 1997). The detection limit and specificity are limited by the poor UV extinction coefficient of the analyte. A derivatization procedure could probably be developed for OA and other lipophilic toxins to allow very sensitive detection with LIF.

8.3.4 Liquid chromatography/mass spectrometry

Since the emergence of atmospheric pressure ionization (API) LC-MS and its first application to marine toxins (Quilliam *et al*., 1989), the technique has proved to be the most powerful analytical tool for these compounds. It is the only technique that allows selective detection and precise quantitation of all known toxins at trace levels in plankton and shellfish. The use of LC-MS also facilitates the identification of new toxins, analogues and metabolites by providing structural information with tandem mass spectrometry (MS/MS) (Quilliam, 1996, 1998; Quilliam *et al*., 2001).

Two basic types of API sources are available: electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). The ESI method has been used more frequently for marine toxins because it is suitable for the ionization of all known toxins. Although APCI has proved useful for some toxins, it is limited in that it requires an analyte to have some volatility and a fair amount of thermal stability. Several different types of mass spectrometers have been used, ranging from single and triple quadrupole MS systems, to ion trap, time-of-flight (TOF) and magnetic sector systems. The various systems have their own advantages and disadvantages, including different sensitivities, and the ability to provide MS/MS for structural information and selective detection. High resolution is a feature of time-of-flight and magnetic sector MS systems that allows the determination of accurate mass and therefore elemental composition of a new toxin.

In this section a few highlights are presented, then a detailed method that allows LC-MS to be used for the simultaneous determination of all lipophilic shellfish toxins is described. Several methods have been reported for LC-MS analysis of OA, DTX1 and DTX2 (Pleasance *et al*., 1990, 1992; Quilliam, 1995; James *et al*., 1997; Draisci *et al*., 1998c; Holmes *et al*., 1999; Suzuki and Yasumoto, 2000). With these methods, a detection limit of 1–10 ng g^{-1} in whole edible shellfish tissue is easily

achieved. Both positive and negative ions can be used with the appropriate mobile phase. It is also possible to directly analyse the diol esters of OA and DTX1, as well as the DTX3 toxins, by this technique (Hu *et al*., 1992*b*; Marr *et al*., 1992). The DTX4- and DTX5-type toxins have also been analysed using negative-ion electrospray LC-MS (Quilliam *et al*., 1996). Specific electrospray LC-MS methods have also been reported for pectenotoxins (Suzuki *et al*., 1998, Suzuki and Yasumoto, 2000), yessotoxins (Draisci *et al*., 1998*a*; Stirling *et al*., in press), azaspiracids (Ofuji *et al*, 1999*b*; Draisci *et al*., 2000), the cyclic imines (Cembella *et al*., 1999; Hu *et al*., 2001; Stirling *et al*., in press), and brevetoxins (Hua *et al*., 1995, 1996). APCI LC-MS methods have also proved useful for the brevetoxins (Dickey *et al*., 1999; Poli *et al*., 2000). Draisci *et al*. (1999) and Goto *et al*. (2001) have recently demonstrated the application of LC-MS to samples containing multiple toxins, including OA and DTX, PTX and YTX. Two different chromatographic conditions were proposed: one for positive-ion detection of OA, DTX and PTX, and another for negative-ion detection of acidic toxins, including YTX. The most important conclusion from all these papers is that LC-MS can be used for all the different lipophilic shellfish toxins.

The main difference in all the above specific methods is the mobile phase, which is the most important factor for achieving success in electrospray LC-MS. Parameters that affect sensitivity include pH, ionic strength, type of buffer, and percentage of the organic solvent. A volatile buffer with a low ionic strength (ideally <10 mM) is most desirable to prevent a build-up of salts on the sampling orifice of the MS. Mobile phases that have been used include aqueous methanol or acetonitrile supplemented with formic acid, acetic acid, trifluoroacetic acid (TFA), ammonium salts of these acids, or ammonium hydroxide. Trifluoroacetic acid has been very popular because it is easy to use and produces a low pH that facilitates protonation of most toxins and deactivation of active sites on silica-based columns. However, TFA forms strong ion pairs with amines and this can lead to suppression of electrospray ionization and reduced sensitivity. It should also be noted that TFA cannot be used if negative-ion work is planned on the same day, as it gives a very strong signal due to the CF_3COO^- anion, which persists in a system for a long time. Similarly, buffers based on amines such as triethylamine should not be used when positive-ion work is planned.

An extensive survey of different mobile phases has been conducted to determine if it is possible to improve LC-MS sensitivity in both positive- and negative-ion modes, and if a wide range of toxins could be analysed in a single analysis (Quilliam *et al*., 2001). A mobile phase based on aqueous acetonitrile with 2 mM ammonium formate and 50 mM formic acid gives a 20- to 50-fold increase in sensitivity compared with TFA in the positive-ion mode and also permits the use of negative-ion detection. Neutral and acidic compounds such as the OA group, pectenotoxins and brevetoxins are ionized in the positive-ion mode as ammonium adduct ions, $[M+NH_4]^+$. Basic compounds such as spirolides and azaspiracids give protonated molecules, $[M+H]^+$. Acidic toxins with sulfate or free carboxyl functions can also be detected in the negative-ion mode as deprotonated molecules, $[M-H]^-$. This mobile phase has been used successfully for over two years and has been found to be suitable for a wide range of compounds in addition to toxins (Quilliam, unpublished data).

Different stationary phases and column dimensions have also been surveyed for work on marine toxins. The goal was to implement rapid gradients for the separation

of a wide range of toxins. Short, narrow-bore columns (50 mm × 2 mm i.d.) packed with 3 µm Hypersil-BDS-C8 phase were found to be the most effective. With this column and the above mobile phase, high-resolution separations are possible. In the analysis illustrated in Fig. 8.11, spirolides, OA and the DTXs (including DTX3), pectenotoxins and azaspiracids were measured. This system has also been successfully used to analyse gymnodimine, yessotoxins, brevetoxins and ciguatoxins. Details of the method are provided in Appendix 8.4 (Quilliam *et al*., 2001). The only problem encountered to date is that some of the heavily sulfated toxins, such as DTX4, do not chromatograph very well at lower pH, due to residual active sites on the stationary phase. Other stationary phases may solve this problem. The main limitation of this multi-toxin LC-MS analysis approach lies in sample preparation. As discussed in Section 8.3.1, it is important to find a universal extraction solvent

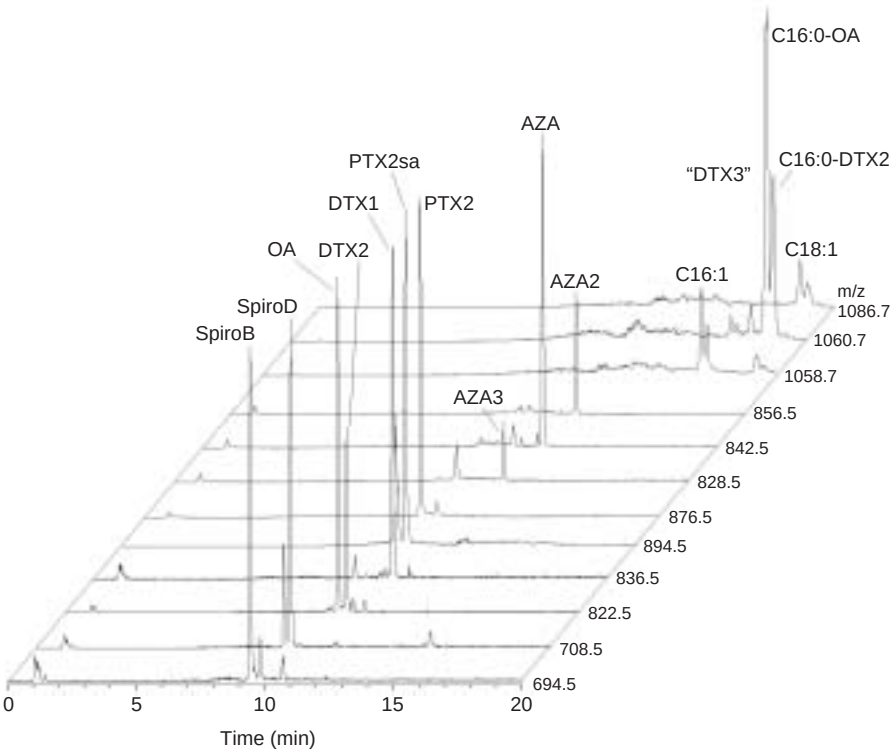


Figure 8.11
Reversed phase gradient elution LC-MS analysis of a wide range of toxins in a blend of contaminated mussel tissue extracts. Selected ion monitoring was performed on either $[M+H]^+$ or $[M+NH_4]^+$ ions, which are displayed as individual mass chromatograms. The toxins present include spirolides (SpiroB, SpiroD), okadaic acid (OA), dinophysistoxins (DTX1, DTX2), pectenotoxins (PTX2, PTX2sa), azaspiracids (AZA), and acyl esters of OA and DTX2 ('DTX3') (conditions given in Appendix 8.4).
Source: Quilliam *et al*. (2001).

and clean-up scheme that can give good recovery for all toxins. The provisional method outlined in Appendix 8.4 has been successfully used in one laboratory but needs to be validated for quantitative work.

In conclusion, LC-MS is probably the most efficient and comprehensive option for the analysis of the lipophilic shellfish toxins. The LC-MS technique meets all the needs of laboratories involved in both monitoring and toxin research: 1) universal detection capability; 2) high sensitivity; 3) high selectivity and specificity; 4) minimal sample preparation; 5) an ability to deal with the structural diversity and labile nature of toxins; 6) separation of complex mixtures of toxins; 7) precise and accurate quantitation; 8) wide linear range; 9) automation; 10) high throughput; 11) rapid method development; 12) legal acceptability for confirmation; and 13) structural information for identification of new toxins, analogues and metabolites. One of the most appealing features of LC-MS to many laboratories is the possibility that a wide range of methods could be replaced by just one instrument.

The lack of readily available calibration standards remains a serious problem for the further development of all chemical methods. With LC-MS, however, it is possible to go a long way without a standard. If the structure of the sought-after analyte is known and a sample or crude extract containing the analyte is available, then one can set up a provisional method in a matter of hours. Use of MS/MS techniques can assist in confirming identities of chromatographic peaks. The method cannot be considered fully quantitative without an accurate standard, but estimates of analyte concentration can be produced by calibrating the instrument with accurate solutions of a closely related compound, where it is fairly safe to assume an equi-molar response (e.g. calibration with OA can allow analysis of DTX1 and DTX2).

The high capital and maintenance costs of LC-MS systems do present difficulties for many laboratories, although the recent introduction of less expensive, easy-to-use 'bench-top' instruments should help to reduce these problems. In addition, the actual cost per sample must be considered carefully when comparing different methods. An analysis by LC-MS can be very rapid (as short as 2–3 min) and can be totally automated, resulting in a very low cost per sample. The cost of LC-MS analyses is also low because minimal sample preparation is required, compared with other analysis methods based on complicated clean-up and derivatization schemes. This feature is also important for sample throughput, as sample preparation is usually the major bottleneck in most laboratories. An additional item of importance to some laboratories is that an LC-MS system can be used for both research and monitoring work, and for a variety of analytes, not just toxins. Obviously the LC-MS option is best suited to a central laboratory that would have the technical expertise and the workload to justify such an instrument. A combination of rapid assay methods for screening and an LC-MS system in a central laboratory for research and confirmation may be the best route to follow.

APPENDIX 8.1

Commercial sources of lipophilic shellfish toxins and certified reference materials

Very few of the lipophilic shellfish toxins are available commercially and, for those that are available, the buyer must be cautious about putting too much faith in the stated quantity or purity of materials provided. In many cases, the materials are sold

primarily for biological/toxicological testing and may not be suitable for quantitative analysis. The following chemical suppliers currently list toxins in their catalogues.

	Supplier code							
Toxin	SIG	CBC	LCL	KBC	WC	RBI	ICN	MP
Okadaic acid (OA)	X	X	X	X	X	X	X	X
Dinophysistoxin-1 (DTX1)	X	X		X	X	X	X	X
Okadaic acid, methyl ester	X	X			X	X	X	X
Brevetoxin-1 (BTX1)	X	X			X	X	X	X
Brevetoxin-2 (BTX2)						X	X	x
Brevetoxin-3 (BTX3)								
Brevetoxin-6 (BTX6)								
Brevetoxin-9 (BTX9)								
Pectenotoxin-6 (PTX6)								

CBC: Calbiochem-Novabiochem International, PO Box 12087, La Jolla, CA 92039-2087, USA; tel: +1 800-854-3417, +1 858-450-9600; fax: +1 858-453-3552; web: www.calbiochem.com

ICN: ICN Pharmaceuticals, Inc., 3300 Hyland Avenue, Costa Mesa, CA 92626, USA; tel: +1 800-854-0530, +1 714-545-0113; fax: +1 800-334-6999; web: www.icnbiomed.com

KBC: Kamiya Biomedical Company, 910 Industry Drive, Seattle, WA 98188, USA; tel: +1 206-575-8068; fax: +1 206-575-8094; web: www.kamiyabiomedical.com

LCL: LC Laboratories, 165 New Boston Street, Woburn, MA 01801, USA; tel: +1 781-937-0777; fax: +1 781-938-5420; web: www.LCLabs.com

MP: Molecular Probes, 4849 Pitchford Ave, PO Box 22010, Eugene, OR 97402-9165, USA; tel: +1 541-465-8300, +1 800-438-2209; fax: +1 541-344-6504; web: http://www.probes.com

RBI: Research Biochemicals International, One Strathmore Road, Natick, MA 01760-2447, USA; tel: +1 508-651-8151, +1 800-736-3690; fax: +1 508-655-1359, +1 800-736-2480; web: www.callrbi.com

SIG: Sigma Chemical Company, PO Box 14508, St Louis, MO 63178-9916, USA; tel: +1 800-325-3010, +1 314-771-5750; fax: +1 314-771-5757; web: www.sigma-aldrich.com

WC: Wako Chemicals USA, Inc., 1600 Bellwood Road, Richmond, VA 23237, USA; tel: +1 800-992-WAKO, +1 804-271-7677; fax: +1 804-271-7791; Wako Pure Chemical Industries, Ltd, 1-2 Doshomachi 3-Chome, Chuo-Ku, Osaka 541, Japan; tel: +81 6-203-3741; fax: +81 6-222-1203; Wako Chemicals GmbH, Nissanstrasse 2, Neuss, D-41468 Germany; tel: +49 2131-311-0; fax: +49 2131-311-100; web: www.wakousa.com

Certified calibration standards and reference materials

An important concern in any analytical method is the availability of accurate calibration standards and reference materials. Marine toxins are expensive, difficult to acquire in high purity, difficult to weigh properly in sub-milligram portions, and toxic. The National Research Council (Canada) Certified Reference

Materials Program (CRMP) is therefore developing certified calibration solutions for marine toxins. The only calibration standard available for lipophilic toxins is OACS–1, a solution of OA in methanol (certified level = 25.3 µg ml^{-1}; each package contains four 0.5 ml ampoules, stable for one year at -12°C in dark conditions).

Certified reference materials are useful for testing both the full implementation of an analytical method and newly developed analytical methods. The CRMP has developed MUS-2, a mussel tissue reference material for okadaic acid and DTX1, which was made by blending mussel digestive glands with a small amount of cultured *Prorocentrum lima* biomass and water, to give an homogenate containing *c*. 11 mg kg^{-1} of OA and 1 mg kg^{-1} of DTX1 (each package contains four 4 g vials, stable in unopened form at -12°C for one year).

These materials may be ordered from CRMP (Institute for Marine Biosciences, 1411 Oxford St, Halifax, NS B3H 3Z1, Canada; tel: +1 902-426-8280; fax: +1 902-426-5426; web: http://www.imb.nrc.ca/crmp_e.html; e-mail: crm.imb@nrc.ca).

APPENDIX 8.2

HPLC-fluorescence analysis of okadaic acid group of toxins

This section presents a detailed procedure for the determination of the DSP toxins OA, DTX1 and DTX2 in shellfish as optimized by Quilliam (1995) with slight modifications. Toxins are extracted from shellfish digestive glands by homogenization with methanol-water (8:2, v/v). Chemical hydrolysis is also performed on a portion of the extract to convert acyl esters to the parent toxins. After an initial clean-up with liquid-liquid partitioning, derivatization with 9-anthryldiazomethane (ADAM), and further clean-up on a silica column, toxins are analysed by HPLC with fluorescence detection.

Reagents and equipment

(a) *Water:* distilled and passed through a water-purification system equipped with ion exchange and carbon filters.

(b) *Solvents:* distilled-in-glass-grade acetonitrile, methanol and hexane; anhydrous ethanol; ACS-grade diethyl ether; ACS-grade chloroform with ethanol preservative.

(c) *Alumina:* Woelm basic alumina (activity grade 1), activated at 450°C overnight (Alupharm Chemicals, New Orleans, LA 70130).

(d) *Silica:* Analytichem Bondesil 40 µm (No. 1221-3001, Varian Sample Preparation Products, Harbour City, CA 90710). Individual 500 mg portions should be measured into small beakers, covered with foil and stored in an oven at 130°C (for at least 24 hours).

(e) *Chloroform (with 1.15% ethanol) for silica clean-up wash step:* activated alumina (50 g) (c) is dry-packed into an oven-dried glass column (35 cm × 21 mm i.d.) with glass frit and Teflon stopcock. ACS-grade chloroform is then passed through the column. The first 10 ml is discarded and the next 50 ml collected into an oven-dried 50 ml volumetric flask containing 575 µl of anhydrous (absolute) ethanol.

(f) *ADAM:* 9-anthryldiazomethane, delivered on dry ice from the supplier (No. A-1400, Molecular Probes Inc., Eugene, OR 97404), should be split immediately into 2 mg portions and stored in amber vials at -80°C.
(g) *ADAM solution:* 2 mg ADAM dissolved in 1 ml methanol. The solution must be prepared daily, used as soon as possible and handled under yellow or subdued lighting.
(h) *7-O-acetylokadaic acid (AcOA, internal standard) (optional):* synthesized by mixing 1 mg okadaic acid, dissolved in a mixture of 100 µl pyridine and 50 µl of 3.2 mg ml^{-1} acetic anhydride in pyridine (Aldrich Chemical Co., Milwaukee, WI 53233), and allowing the reaction to proceed at room temperature overnight. After evaporation and redissolution in methanol, the major product (AcOA) is isolated by preparative HPLC using a Vydac 201TP column (250 mm × 10 mm i.d.) with 4 ml min^{-1} methanol-water (6:4, v/v) and UV detection at 220 nm.
(i) *AcOA (internal standard) solution:* 100 µg AcOA dissolved in 1 ml methanol.
(j) *Deoxycholic acid (DCA):* 98% purity (No. D2510, Sigma Chemical Co., St Louis, MO 63178).
(k) *DCA solution:* 3.5 mg deoxycholic acid dissolved in 100 ml methanol.
(l) *Okadaic acid (OA):* 99% purity (Diagnostic Chemicals Ltd, Charlottetown, PEI, Canada).
(m) *OA solution:* OACS–1 certified calibration solution (25.3 µg ml^{-1}, CRMP, National Research Council (Canada), 1411 Oxford St, Halifax, NS B3H 3Z1, Canada). Perform accurate dilutions of OACS–1 in methanol to give solutions of concentrations 1.0, 2.5, 5.0 and 12.5 µg ml^{-1}.
(n) *Calibration solution:* mix exactly 400 µl of OA solution (n) (either OACS–1 or one of the accurate dilutions of OACS–1 in methanol), 140 µl DCA solution (l), 50 µl AcOA solution (j) and 110 µl methanol in a 1.5 ml amber glass screw-cap vial (if AcOA solution is not available, the methanol should be increased to 160 µl).
(o) *Mussel tissue reference material:* MUS-2 (CRMP).
(p) *Empty SPE tubes:* 7 ml glass barrels and Teflon frits (No. 7999BJ, Baxter Healthcare Corp., Burdick & Jackson Division, Muskegon, MI 49442).
(q) *Derivatization vial:* screw cap amber glass vial, 1.5 ml capacity, Teflon-lined cap (No. C221200A, Chromatographic Specialties, Brockville, Ontario). Vials should be cleaned thoroughly with acetone and dried at 70°C overnight.
(r) *Liquid chromatograph:* isocratic system able to generate >2,500 psi and equipped with a dual monochromator fluorescence detector with 254 nm excitation and 412 nm emission protected by a 280 nm cut-off filter. A filter fluorescence detector with the appropriate bandpass filters can also be used.
(s) *HPLC column:* stainless steel, 25 cm × 4.0 mm i.d., packed with 5 µm LiChrospher–100 RP18 octadecylsilica (Merck, Darmstadt, Germany).

Preparation of samples

1. *Tissue preparation:* Tissues should be drained after removal from shellfish. Separate and pool the digestive glands (hepatopancreas) and other soft tissues from several animals in a lot. Weigh the pooled tissues to determine the fraction of whole edible tissue represented by digestive glands. For representative sampling, approximately 20 g of digestive glands pooled from several animals

should be homogenized in a blender. Subsampling from this homogenate should be done immediately after blending while still well mixed or later after mixing again. If an analysis must be performed on a limited amount of sample, a portion of chopped or ground tissue can be weighed directly into the extraction tube.

2. *Extraction:* Weigh a 2.0 g portion of homogenized tissue sample accurately into a 50 ml plastic centrifuge tube. Add an accurate 100 µl aliquot of AcOA (internal standard) solution (j) and 7.9 ml of methanol-water (8:2, v/v). If AcOA is not available use 8.0 ml extraction solvent instead. Homogenize the mixture using a Polytron mixer for 3 min at 6–10 K rpm. Sample adhering to the probe may be shaken into the extraction tube but no additional solvent should be used to recover the sample residues; the probe should be washed separately before use on the next sample. Centrifuge the sample at 4,000 × *g* or higher for 10 min. Decant the supernatant into a screw-cap vial and store in the freezer until step 3 is executed.
3. *Hydrolysis of acyl esters of OA and DTX:* Mix an accurate 2.5 ml aliquot of crude extract (supernatant from step 2) with 2.5 ml of 0.5 M NaOH in methanol-water (8:2, v/v) in a 15 ml glass, screw-cap centrifuge tube. Seal the tube and heat at 35°C for 40 min. After this time, add 100 µl of concentrated HCl (11.6 M) and mix.
4. *Liquid-liquid partitioning clean-up:* Transfer an accurate 2.5 ml aliquot of crude extract (supernatant from step 2) to a 15 ml glass centrifuge tube and mix with 2.5 ml of methanol-water (8:2, v/v). For the hydrolysate from step 3, use the entire solution (*c*. 5 ml) already in a 15 ml glass centrifuge tube, without addition of more solvent. Extract twice with 5 ml aliquots of n-hexane by vortex mixing for 0.5 min. After discarding the hexane layers, add 1 ml of water with 0.2% acetic acid and 6 ml chloroform to the tube and vortex mix the mixture for 0.5 min. Transfer the lower chloroform layer to a 50 ml glass test tube. Repeat the extraction of the aqueous layer with another 6 ml of chloroform. Evaporate the combined chloroform layers to dryness under a stream of nitrogen. Dissolve the residues after evaporation in exactly 100 µl DCA solution (l) plus 400 µl methanol by vortex mixing. Transfer this solution (without further addition of solvent) to a 1.5 ml amber glass screw-cap vial. The dissolution and transfer operations must be done quickly to avoid evaporation of the methanol. This extract contains 1 g digestive gland equivalent per millilitre of solution.
5. *ADAM derivatization:* Transfer 35.0 µl aliquots of cleaned sample extracts (from step 4), calibration solution (o), or methanol (reaction blank) into individual cleaned 1.5 ml amber vials. Working under yellow or subdued light, add 100 µl aliquots of 0.2% (w/v) ADAM solution (h) to each of the vials. After sealing tightly with a Teflon-lined screw cap, sonicate the solutions for 10 min in warm water (37°C) and then maintain at 37°C for 2 hours in the dark. Finally, use a vacuum centrifuge or nitrogen stream to evaporate all reaction solutions to dryness.
6. *Silica clean-up:* Place clean, dry glass SPE tubes (7 ml capacity) equipped with Teflon frits on an SPE vacuum manifold and pack with 500 mg of activated silica (d) (this operation should be done immediately prior to use in order to avoid deactivation of the silica by atmospheric moisture). Condition the columns with 6 ml ACS-grade chloroform followed by 3 ml chloroform-hexane (1:1). Stop the flow when the meniscus reaches the top of the packing; the

columns should not be allowed to go dry thereafter. Redissolve residues from the evaporated ADAM reactions (from step 5) and transfer to the columns using three 300 µl aliquots of chloroform-hexane (1:1) and pass slowly (1 drop s^{-1}) through to waste. Wash the columns with 5 ml chloroform-hexane (1:1) and 5 ml alumina-cleaned chloroform containing 1.15% ethanol (f). After placing clean glass test tubes under each column, elute the ADAM derivatives with 5 ml methanol-chloroform (1:9). Evaporate the eluates to dryness under a nitrogen stream and then dissolve the residues in exactly 500 µl methanol. Transfer these solutions (without further addition of solvent) to amber vials for HPLC analysis.

HPLC determination

Analyse derivatized, cleaned samples from step 5 by HPLC using isocratic conditions with a mobile phase of 1.0 ml min^{-1} acetonitrile-water (8:2, v/v), the analytical column maintained at 40°C, and an injection volume of 10 µl. If a column heater is not available, ambient temperature may be used with the percentage of acetonitrile in the mobile phase adjusted to 85–90%. The fluorescence detector should be set for 254 nm excitation and 412 nm emission protected by a 280 nm cut-off filter (wavelength accuracy should be checked). A full calibration curve experiment should be run at least once using the entire set of derivatized calibration solutions (o) to ensure linearity and zero intercept. If good linearity and zero-point intercept are evident, a single-point calibration may be performed thereafter by analysing one calibration solution with a concentration similar to that of the samples. Replicate injections should have CVs <5%. The reaction blank should show no interfering peaks at the retention times of the derivatized toxins. Inject all samples in duplicate. Avoid carryover between injections of different samples by washing the injector loop. Average peak areas for each sample. Repeat single injections of calibration standard every 2 hours and duplicate injections every 8 hours. In calculations, average the peak areas of standards immediately following and preceding a series of samples.

Calculate the concentration of OA, DTX1 or DTX2 (mg kg^{-1}) in each sample according to equation [1], where A_U is the average peak area for the toxin in the sample, A_S is the average area for OA in the calibration standards (concentration C_S µg ml^{-1}) bracketing the sample, A_{IU} is the average peak area for the internal standard (AcOA) in the sample, A_{IS} is the average area for the internal standard in the calibration standard, W is the weight in grams of digestive glands extracted (*c*. 2.0 g), and F is the fraction of the whole tissue represented by the digestive glands.

$$\text{Toxin concentration (mg kg}^{-1}\text{ whole tissue)} = 2(A_U/A_S)(A_{IS}/A_{IU})(C_S/W)(F) \quad [1]$$

The second internal standard (DCA) is only used to determine the effectiveness of the derivatization reaction. Incomplete derivatization may occur due to an impure reagent and/or excessive matrix interference. The AcOA internal standard will correct for as much as 50% incomplete derivatization as it reacts at exactly the same rate as the toxins. The DCA reacts more slowly than the toxins, however, and is therefore more sensitive to matrix effects. The peak areas for the DCA internal standard in both samples and standards should be within 10% of each other for quantitation by the second equation to be valid.

If AcOA is not available as an internal standard, equation [2] should be used.

Toxin concentration (mg kg^{-1} whole tissue) = $2(A_U/A_S)(C_S/W)(F)$ [2]

The difference between the hydrolysed and non-hydrolysed extracts represents the amount of OA or DTX1 that is present in the sample as acyl esters.

APPENDIX 8.3

HPLC-fluorescence analysis of yessotoxins

This section presents a procedure developed by Yasumoto and Takizawa (1997) for the LC-FLD determination of the yessotoxin group of toxins in shellfish. Toxins are extracted from shellfish tissues by homogenization with methanol-water (8:2, v/v). After a C18-silica solid phase extraction (SPE) clean-up, the analytes are derivatized with the reagent DMEQ-TAD, cleaned again with C18-SPE, and analysed by LC-FLD.

Reagents and equipment

(a) *Water:* distilled and passed through a water-purification system equipped with ion exchange and carbon filters.
(b) *Reagents:* distilled-in-glass-grade acetonitrile and methanol; ACS-grade chloroform, dichloromethane; reagent-grade $Na_2HPO_4.12H_2O$ and $NaHPO_4.2H_2O$; DMEQ-TAD, available from Wako Pure Chemical Industries (Osaka), was used as a 0.1% solution in dichloromethane.
(c) *SPE cartridge:* Sep-pak plus C18 short body cartridge columns (Waters, USA), washed with 6 ml 20 mM phosphate buffer (pH 5.8) before equilibration with solvents to be used.
(d) *Liquid chromatograph:* isocratic system equipped with dual monochromator fluorescence detector.
(e) *HPLC column:* Cosmosil 5C18AR column (4.6 mm × 250 mm, Nacalai Tesque, Kyoto).
(f) *Mobile phase A:* mixture of 3 parts 40 mM phosphate buffer (pH 5.8) and 7 parts methanol.
(g) *Mobile phase B:* mixture of 4 parts 35 mM phosphate buffer (pH 5.8) and 6 parts methanol.

Preparation of samples

1. *Tissue preparation:* Isolate and homogenize digestive glands as in Appendix 8.2.
2. *Extraction:* Homogenize 1.0 g tissue with 9 ml methanol-water (8:2, v/v) for 3 min. Centrifuge the homogenate at 3,000 rpm for 10 min and decant the supernatant into a storage vial. Mix 0.5 ml extract with 1.5 ml 20 mM phosphate buffer (pH 5.8) and pass through a C18-SPE cartridge. After washing with 6 ml methanol-water (2:8), elute the analyte with 6 ml methanol-water (7:3). Evaporate the eluent to dryness.
3. *Derivatization:* React the residue with 50 µl 0.1% DMEQ-TAD in dichloromethane at room temperature for 2 hours in the dark. Add 50 µl methanol to quench the reaction and then evaporate the mixture to dryness.

4. *Clean-up:* Dissolve the residue in methanol-water (3:7), load on to a C18-SPE cartridge, wash with 8 ml methanol-water (3:7), and elute with methanol-water (7:3). Evaporate the eluent to dryness and then dissolve in 50 µl methanol.

HPLC determination

Analyse derivatized, cleaned samples from step 4 by HPLC using isocratic conditions with 1.0 ml min^{-1} mobile phase A for YTX and 45-OH-YTX or mobile phase B for trinor-YTX. Maintain the HPLC column at 35°C and use an injection volume of 10 µl. The fluorescence detector should be set for 370 nm excitation and 440 nm emission.

APPENDIX 8.4

LC-MS analysis of lipophilic shellfish toxins

This section presents a procedure for the use of LC-MS to screen shellfish tissues for the presence of assorted lipophilic shellfish toxins (Quilliam *et al*., 2001). Toxins are extracted from shellfish tissues by homogenization with methanol-water (8:2, v/v). After a liquid-liquid partitioning into chloroform, solvent evaporation and dissolution in methanol, toxins are analysed directly by LC-MS using selected ion monitoring.

Reagents and equipment

(a) *Water:* distilled and passed through a water-purification system equipped with ion exchange and carbon filters.
(b) *Solvents:* distilled-in-glass-grade acetonitrile and methanol; ACS-grade chloroform.
(c) *Liquid chromatograph:* system should be capable of performing gradient elution with 0.2 ml min^{-1} flow rates.
(d) *HPLC column:* Keystone Scientific (Bellefonte, PA) Quicksilver cartridge column (50 mm × 2 mm) packed with 3 µm Hypersil-BDS-C8 and equipped with a guard column.
(e) *Mobile-phase buffer stock solution:* 1 M formic acid and 40 mM ammonium formate in water.
(f) *Mobile phase A:* dilute 1 part buffer stock solution (e) with 19 parts water.
(g) *Mobile phase B:* dilute 1 part buffer stock solution (e) with 19 parts acetonitrile.
(h) *Mass spectrometer:* system should be capable of nebulizer-assisted electrospray ionization and selected ion monitoring (SIM) data acquisition.

Preparation of samples

1. *Tissue preparation:* Edible tissues should be removed from shellfish and drained. For representative sampling, approximately 100 g of tissues pooled from several animals should be homogenized in a blender. Subsampling from this homogenate should be done immediately after blending while still well mixed or later after mixing again. If an analysis must be performed on a limited amount of sample, a portion of chopped or ground tissue can be weighed directly into the extraction tube.

2. *Extraction:* Weigh a 2.0 g portion of homogenized tissue sample accurately into a 50 ml plastic centrifuge tube. Add 8.0 ml methanol-water (8:2, v/v) and homogenize the mixture using a Polytron mixer for 3 min at 6–10 K rpm. Sample adhering to the probe may be shaken into the extraction tube but no additional solvent should be used to recover the sample residues; the probe should be washed separately before use on the next sample. Centrifuge the sample at 4,000 × *g* or higher for 10 min. Decant the supernatant into a screw-cap vial and store in the freezer until step 3 is executed.
3. *Liquid-liquid partitioning:* Transfer an accurate 5 ml aliquot of crude extract (supernatant from step 2) to a 15 ml glass centrifuge tube. Add 1 ml of water with 0.2% acetic acid and 6 ml chloroform to the tube, and vortex mix for 0.5 min. Transfer the lower chloroform layer to a glass test tube. Repeat the extraction of the aqueous layer with another 6 ml of chloroform. Evaporate the combined chloroform layers to dryness under a stream of nitrogen. Dissolve the residues after evaporation in 0.5 ml methanol by vortex mixing. Transfer this solution (without further addition of solvent) to a 1.5 ml amber glass screw-cap vial via a 0.4 µm filter. The dissolution and transfer operations must be done quickly to avoid evaporation of the methanol. This extract contains 2 g tissue equivalent per millilitre of solution.

LC-MS determination

The LC-MS analysis is performed using a binary gradient LC with mobile phases A and B [see (f) and (g) in this section] and a 50 mm × 2 mm Hypersil-BDS-C8 column. Gradient elution from 5% to 100% B is performed over 10 min and then held at 100% B for 10 min, before returning to initial conditions and equilibrating for 7 min. The flow rate is 0.2 ml min^{-1} and the injection volume is 5 µl. The ions in Table 1 should be used for selected ion monitoring (SIM) acquisition. If a tandem mass spectrometer is available, selected reaction monitoring (SRM) techniques can be used to gain increased selectivity.

TABLE 8.1 List of ions to be monitored in the LC-MS analysis of assorted lipophilic shellfish toxins

	Positive-ion mode		**Negative-ion mode**	
Toxin	$[M+NH_4]^+$	$[M+H]^+$	$[M-H]^-$	$[M-2H]^{-2}$
	m/z	m/z	m/z	m/z
OA, DTX2	822.5	805.5	803.5	na
DTX1	836.5	819.5	817.5	na
C14:0-OA, -DTX2	1032.7	1015.7	1013.7	na
C16:1-OA, -DTX2	1058.7	1041.7	1039.7	na
C16:0-OA, -DTX2	1060.7	1043.7	1041.7	na
C18:1-OA, -DTX2	1086.7	1069.7	1067.7	na
C20:5-OA, -DTX2	1106.7	1089.7	1087.7	na
C22:6-OA, -DTX2	1132.7	1115.7	1113.7	na
C14:0-DTX1	1046.7	1029.7	1027.7	na
C16:1-DTX1	1072.7	1055.7	1053.7	na
C16:0-DTX1	1074.7	1057.7	1055.7	na
C18:1-DTX1	1100.7	1083.7	1081.7	na
C20:5-DTX1	1120.7	1103.7	1101.7	na
C22:6-DTX1	1146.7	1129.7	1127.7	na
PTX2	876.5	859.5	na	na
PTX1, PTX4, PTX8	892.5	875.5	na	na
PTX6, PTX7, PTX9	906.5	889.5	887.5	na
PTX2sa, *epi* -PTX2sa	894.5	877.5	875.5	na
AZA1	na	842.5	840.5	na
AZA2	na	856.5	854.5	na
AZA3	na	828.5	826.5	na
Spirolide A, desMeC	na	692.5	na	na
Spirolide B	na	694.5	na	na
Spirolide C	na	706.5	na	na
Spirolide D	na	708.5	na	na
Spirolide E	na	710.5	na	na
Spirolide F	na	712.5	na	na
Gymnodimine	na	508.3	na	na
Gymnodimine B	na	524.3	na	na
BTX1	884.5	867.5	na	na
BTX7	886.5	869.5	na	na
BTX10	888.5	871.5	na	na
BTX2	912.5	895.5	na	na
BTX3	914.5	897.5	na	na
BTX9	916.5	899.5	na	na
BTX-B1	1035.5	1018.5	1016.5	na
BTX-B2	1051.5	1034.5	1032.5	na
YTX	1160.5	1143.5	1141.5	570.3
Homo-YTX	1174.5	1157.5	1155.5	577.3
45-hydroxy-YTX	1176.5	1159.5	1157.5	578.3
45-hydroxyhomo-YTX	1190.5	1173.5	1171.5	585.3
Adriatoxin	1068.5	1051.5	1049.5	524.3

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9

Chemical methods for domoic acid, the amnesic shellfish poisoning (ASP) toxin

M. A. Quilliam

9.1 Introduction

The amnesic shellfish poisoning (ASP) toxin, domoic acid, was originally isolated from a red macroalga *Chondria armata* by Japanese researchers studying the insecticidal properties of algal extracts (Takemoto and Daigo, 1958). The structure was later revised by Ohfune and Tomita (1982) (see Fig. 9.1). Domoic acid belongs to a group of amino acids called the kainoids, which are classed as neuroexcitants or excitotoxins that interfere with neurotransmission mechanisms in the brain. The original member of the group, kainic acid (Fig. 9.1), was isolated earlier from another red macroalga *Digenea simplex* (Murakami *et al*., 1953). Other members of the group include acromelic acids A and B, isolated from mushrooms (Konno *et al*., 1988).

The ability to perform quantitative analyses for domoic acid became very important after the 1987 incident of ASP in eastern Canada (Wright *et al*., 1989). Domoic acid was shown to be the causative agent for that incident and was found to be present in the shellfish at levels as high as 1,000 mg kg^{-1} in tissues that caused the illness. This contamination level was easily detected by mouse bioassay for paralytic shellfish poisoning (PSP) (AOAC …, 2000*a*), with symptoms of domoic acid toxicity being very characteristic and clearly distinct from those for PSP toxins. It quickly became apparent, when the regulatory level was set at 20 mg kg^{-1} by Canadian regulatory authorities, that the mouse bioassay with a detection limit for domoic acid of only 40 mg kg^{-1} could not be used for routine monitoring. High-

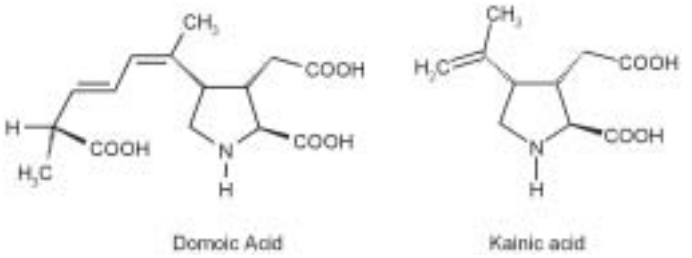

Figure 9.1
Structures of domoic acid and kainic acid.

performance liquid chromatography with ultraviolet detection (HPLC-UV) was the first chemical analytical method for domoic acid and is still the most commonly used for monitoring shellfish. This method has the advantages of being relatively simple, fast, reproducible and accurate. It has also been validated through the AOAC International Official Methods Program (AOAC …, 2000*b*), which gives it increased acceptance by the regulatory community. A number of other analytical approaches, including thin-layer chromatography (TLC), capillary electrophoresis (CE) and liquid chromatography with detection by mass spectrometry (LC-MS), have also been developed and are presented in this chapter.

Studies immediately after the ASP incident in 1987 led to the discovery that domoic acid was produced by the diatom *Pseudo-nitzschia multiseries* (= *Nitzschia pungens* f. *multiseries*) (Subba Rao *et al*., 1988; Bates *et al*., 1989). A laboratory culture of this diatom produced domoic acid at levels ranging from 1–20 pg $cell^{-1}$, with <1 ng ml^{-1} found in the culture medium (Bates *et al*., 1991). As the sensitivity of the HPLC-UV method was inadequate for the analysis of most plankton and seawater samples, more sensitive techniques were required for experiments involving such components (Pocklington *et al*., 1990). Thus, methods based on derivatization with fluorescence-reagents, such as 9-fluorenylmethyl-chloroformate, and subsequent analysis by HPLC with fluorescence detection (FD) are important for the quantitative analysis of domoic acid in toxin production studies.

Although the chemical analysis of domoic acid is not difficult, in certain critical situations detailed confirmation of toxin identity is paramount. For example, when attempting to establish whether or not domoic acid has appeared in a new geographical region or is present in another plankton species, it is not adequate to simply report that a peak in an HPLC chromatogram has the same retention time as that of a domoic acid standard. The acquisition of spectroscopic data such as ultraviolet and mass spectra provides a much higher degree of certainty that the putative toxin has been correctly identified. Therefore, techniques such as HPLC combined with ultraviolet diode array detection (UV-DAD) or mass spectrometry (MS) are very important for a research laboratory performing such confirmatory investigations.

Rapid assay techniques (see Chapter 12) will be used increasingly to screen samples for the presence of domoic acid and other toxins. As long as such methods have a low false negative rate, they can be very effective at protecting public health and reducing costs. However, it will also be essential to retain chemical analysis methods to confirm the presence of domoic acid in 'positive' samples as the legal implications of declaring a toxic incident are significant. Analytical methods can also yield a higher degree of accuracy and precision than most assay methods, so they will continue to play an important role in research on domoic acid.

9.2 Chemistry

9.2.1 Chemical and physical properties

A considerable amount of chemical data for domoic acid is available (Takemoto and Daigo, 1958, 1960; Ohfune and Tomita, 1982; Wright *et al*., 1989). The toxin is a crystalline water-soluble compound displaying properties typical of an acidic amino acid. Certain physical-chemical properties of domoic acid are shown in Table 9.1.

TABLE 9.1 Physical-chemical properties of domoic acid

Melting point	Optical rotation	Mol. weight	UV (ethanol)	IR (film) (cm^{-1})
215–216°C (dihydrate)	$[\alpha]_D^{25}$ – 120.5° (anhydrous)	$C_{15}H_{21}NO_6$ 311.14	242 nm $\varepsilon = 2.43 \times 10^4$ (pH 2) $\varepsilon = 2.61 \times 10^4$ (pH 7)	3 500–2 500, 1 715, 1 400, 1 215, 966
	$[\alpha]_D^{25}$ – 108° (dihydrate)			

The spectral data from UV (Falk *et al*., 1989), infrared (IR) (Falk 1988), and nuclear magnetic resonance (NMR) (Walter *et al*., 1992) analysis of domoic acid are pH-dependent, and five protonated forms of the toxin are possible. The carboxyl group at C2 is most acidic, followed by that at C5′ and finally the C7 group.

Secondary amino acids such as proline, kainic acid and domoic acid form a yellow derivative with ninhydrin. The colour is distinct enough for the qualitative detection of domoic acid in mixtures or hydrolysates separated by thin-layer or paper chromatography. The utility of other colour reagents has been investigated, and although both isatin and vanillin give well-developed coloured derivatives with domoic acid, the vanillin stain was found to be particularly characteristic for domoic as well as kainic acid (Dallinga-Hannemann *et al*., 1995).

A detailed stability study on domoic acid has been conducted (Thomas *et al*., 2001). Extensive decomposition was observed when aqueous solutions were stored either at high temperatures (50°C) or at extremes of pH (2 or 12), or when exposed to light or oxygen. At high temperatures, the major decomposition product was 5′-epi-domoic acid. Surprisingly, significant but variable levels of decomposition were observed in aqueous solutions stored at -12°C in a conventional freezer. These results are believed to be due either to the incomplete freezing of the solution, or to a freeze/thaw cycle induced by poor temperature control. Long-term storage in aqueous methanol can lead to some formation of methyl esters. For maximum stability, domoic acid solutions should be stored at pH 5–7, under argon or nitrogen, and in the dark. For storage of up to a year +4°C is acceptable, but for long-term storage it is best to store at -80°C. Aqueous solutions should not be stored in conventional freezers at -12°C under any conditions. The effect of storage temperature on domoic acid in a certified mussel tissue reference material has also been investigated. At high temperatures (50°C), in addition to conversion to 5′-epi-domoic acid, there was extensive decomposition to undetectable products, possibly due to covalent binding to proteins and other materials in the mussel tissue. Mussel tissues containing domoic acid should be kept at -80°C for long-term storage but they appear to be stable at -12°C for several months. If the tissues have been thermally sterilized, they can be stored at +4°C for several months.

9.2.2 Naturally occurring isomers

Investigation of insecticidal kainoids present in the red alga *Chondria armata* resulted in the discovery, in minor amounts, of the isomers isodomoic acid A, B and C (Maeda *et al*., 1986) (see Fig. 9.2) as well as domoilactones A and B (Maeda *et al*., 1987). Recently, isodomoic acids G and H have also been identified in *Chondria armata* (Zaman *et al*., 1997). Domoic acid is the major congener present in plankton

or contaminated shellfish, although three geometrical isomers (isodomoic acids D, E and F) and 5′-epi-domoic acid (Fig. 9.2) have been isolated in small amounts from both plankton cells and shellfish tissue (Wright *et al*., 1990; Walter *et al*., 1994). The geometrical isomers can be prepared in the laboratory by brief exposure of dilute solutions of domoic acid to UV light (254 nm), and are not considered to be *de novo* products of the plankton. Pharmacological studies indicate that these photoisomers bind less strongly to the kainate receptor proteins than domoic acid, suggesting that they are not as toxic as the parent amino acid (Hampson *et al*., 1992). Formation of the 5′-epimer is accelerated with warming (Quilliam, unpublished data) and (importantly) this epimer shows almost the same binding efficacy to the kainate receptor as domoic acid (Hampson *et al*., 1992). All of these isomers can be separated from domoic acid by the HPLC method described in this chapter.

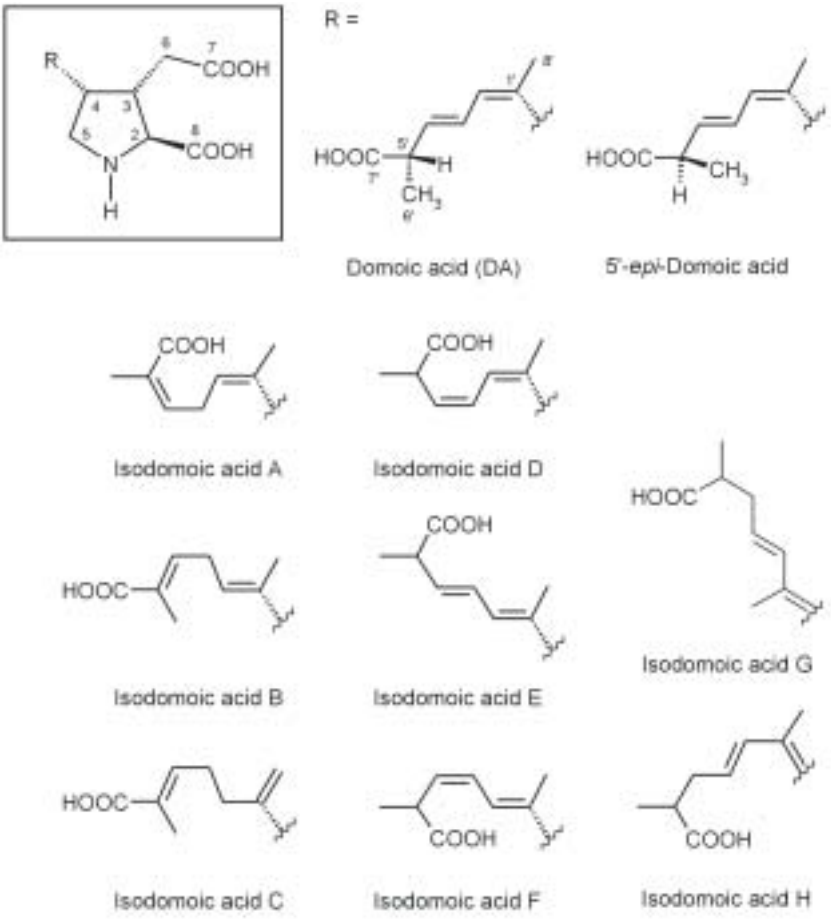

Figure 9.2
Structures of domoic acid isomers.

9.3 Chemical analysis

9.3.1 Extraction and clean-up methods

Domoic acid was first extracted from contaminated shellfish using the standardized procedure for mouse bioassay of PSP toxins (AOAC …, 2000*a*). This involves boiling drained shellfish tissue with an equal volume of 0.1 N HCl and then filtering a portion of the supernatant (see Chapter 13). This procedure provides an extract suitable for mouse bioassay and HPLC analysis of both PSP toxins and domoic acid, therefore it was adopted with slight modifications for routine regulatory analysis (Lawrence *et al*., 1989, 1991; AOAC …, 2000*b*). There are some problems with this procedure: (a) partial decomposition of domoic acid may occur during the extraction, especially at trace levels; (b) there may be difficulty with certain matrices (e.g. anchovy tissues tend to form a gel); (c) a long sample-preparation time is required; (d) extracts are not very stable (due to decomposition of domoic acid in the acidic solution); and (e) HPLC analytical columns have a short lifetime because of the lack of sample clean-up.

For the initial structural characterization, preparative isolation of domoic acid from mussel tissues was accomplished with an aqueous 50% methanol extraction (Wright *et al*., 1989). Alternative extraction methods, such as aqueous methanol versus boiling water, were also investigated by Quilliam and co-workers (1989*a*) for quantitative determinations. Whereas both solvents gave reproducible, high recoveries, the boiling-water procedure was recommended at the time because it was compatible with a C18 solid phase extraction (SPE) clean-up. The latter step was useful for extending the lifetimes of HPLC columns. Recently, a rapid extraction and clean-up procedure was developed for shellfish and finfish tissues based on an extraction with water-methanol (1:1) and clean-up by strong anion exchange (SAX) SPE (Quilliam *et al*., 1995). With this procedure, the recovery of domoic acid is >90% even at trace levels. The crude methanolic extract is cleaner than aqueous extracts, but must be diluted 5-fold with water before HPLC analysis to avoid appreciable peak broadening due to the 'solvent wash-out' effect. Under the conditions specified (see Appendix 9.2), the SAX clean-up provides unique selectivity towards the strongly acidic domoic acid, thus eliminating most interferences in the HPLC analysis. This clean-up procedure also provides a pre-concentration that facilitates trace level detection of domoic acid.

9.3.2 Thin-layer chromatography

Domoic acid can be analysed semi-quantitatively by TLC methods (Quilliam *et al*., 1998). This should be useful for laboratories not equipped with an HPLC system. Domoic acid can be detected on TLC plates (silica gel 60 F_{254}) as a weak UV-quenching spot that stains yellow after spraying with a 1% solution of ninhydrin. Normal amino acids that are present in crude extracts will interfere and must be separated from domoic acid. With plankton samples, this can be accomplished using two-dimensional TLC. Crude extracts of shellfish tissues cannot be analysed directly, as they are too complex. The SAX-SPE clean-up method, with the minor modification described in Appendix 9.2 (step 6), yields fractions that can be used directly or concentrated *in vacuo* before applying to a silica-gel plate. Only one-dimensional TLC is required with this clean-up as almost all the interfering amino

acids are removed (see Appendix 9.2). The detection limit for domoic acid is about 0.5 µg by this method, which permits detection in shellfish tissues at about 10 mg kg^{-1}.

Domoic acid can also be detected on TLC plates using other spray reagents mentioned previously (Dallinga-Hannemann *et al*., 1995). For example, after spraying a TLC plate with vanillin, a yellow colour forms first, which changes to pink over time when domoic (or kainic) acid is present.

9.3.3 Amino-acid analysis

Crude aqueous extracts of plankton can be analysed directly for domoic acid by an amino acid analyser system (Laycock, personal communication). Using the buffer solutions and ion-exchange column normally used for the analysis of protein hydrolysates, domoic acid elutes close to methionine. Absorbance measurement at 440 nm provides detection of amino acids with primary amine groups, while absorbance at 570 nm selectively detects imino acids such as proline and domoic acid. The detection limit of this method for domoic acid is about 1 µg ml^{-1}, with about 50 µl of extract injected on-column. Although the sensitivity of the amino acid analysis method is close to that of the HPLC-UV method, it is not as effective for samples containing a high concentration of free amino acids and the analysis time is much longer. Shellfish extracts can also be analysed by this approach after the necessary clean-up and concentration of material, as described above.

9.3.4 High-performance liquid chromatography

In early studies on domoic acid in plankton and shellfish, domoic acid was analysed, as well as preparatively isolated, either by high-performance liquid chromatography or ion-exchange chromatography using UV absorbance detection (Wright *et al*., 1989). Reversed-phase HPLC was found to yield the fastest and most efficient separations. Use of an acidic mobile phase to suppress ionization of the carboxyl functions is recommended, and selective separation of domoic acid and its isomers is best achieved with 'polymeric-like' octadecylsilica phases such as Vydac 201TP (Quilliam *et al*., 1989*a*).

The HPLC-UV method, which has been used since 1987 in regulatory laboratories, is the preferred analytical technique for the determination of domoic acid in shellfish (Quilliam *et al*., 1989*a*; Lawrence *et al*., 1989, 1991; AOAC …, 2000*b*). The detection of domoic acid is facilitated by its strong absorbance at 242 nm. The HPLC-UV detection limit for domoic acid is about 10–80 ng ml^{-1}, depending on the sensitivity of the UV detector. The detection limit in tissue is dependent upon the method of extraction and clean-up. If crude extracts (either acidic or aqueous methanol) are analysed without clean-up, the practical limit for quantitation is about 1 mg kg^{-1} (ppm). This is suitable for most regulatory laboratories concerned with detecting contamination levels greater than 20 mg kg^{-1}. However, interferences are commonly encountered that can give false positives with crude extracts. For example, tryptophan and some of its derivatives are often present in substantial concentrations in shellfish and finfish tissues and these compounds elute close to domoic acid (Quilliam *et al*., 1989*a*). A photodiode array detector (DAD) can be used to examine UV spectra to confirm the presence of domoic acid, but this option may not always be available. Fig. 9.3 shows HPLC-UV chromatograms for crude aqueous methanol

extracts of three mussel tissues with 0 mg kg^{-1}, 2 mg kg^{-1} and 20 mg kg^{-1} domoic acid. A large peak due to tryptophan (marked T) is apparent in the chromatograms. Under these HPLC conditions, it does not interfere with domoic acid but it does coelute with one of the domoic acid isomers. With the combined aqueous methanol extraction, SAX-SPE clean-up and HPLC-UV analysis procedure detailed in Appendix 9.2, the detection limit is 20–30 µg kg^{-1} (ppb) and the chromatograms are free from such interferences. Fig. 9.4 shows the analysis of the same three samples shown in Fig. 9.3 after clean-up with the SAX-SPE procedure. The enhanced detectability due to preconcentration and the elimination of tryptophan due to selective clean-up are clearly apparent in these chromatograms.

Another example of HPLC analysis is presented in Fig. 9.5A, which shows the use of a gradient elution separation combined with the UV-DAD for the confirmation of domoic acid (80 mg kg^{-1}) in a razor clam sample. The inset (Fig. 9.5B) shows the UV spectrum acquired for the domoic acid peak, with the characteristic 242 nm absorbance maximum.

A very sensitive procedure, based on reaction with 9-fluorenylmethylchloroformate to form the fluorenylmethoxycarbonyl (FMOC) derivative and HPLC analysis with fluorescence detection, has been developed for monitoring of domoic acid in marine matrices such as seawater and phytoplankton (Pocklington *et al*., 1990). The detection limit is as low as 15 pg ml^{-1} for domoic acid in seawater. Appendix 9.3

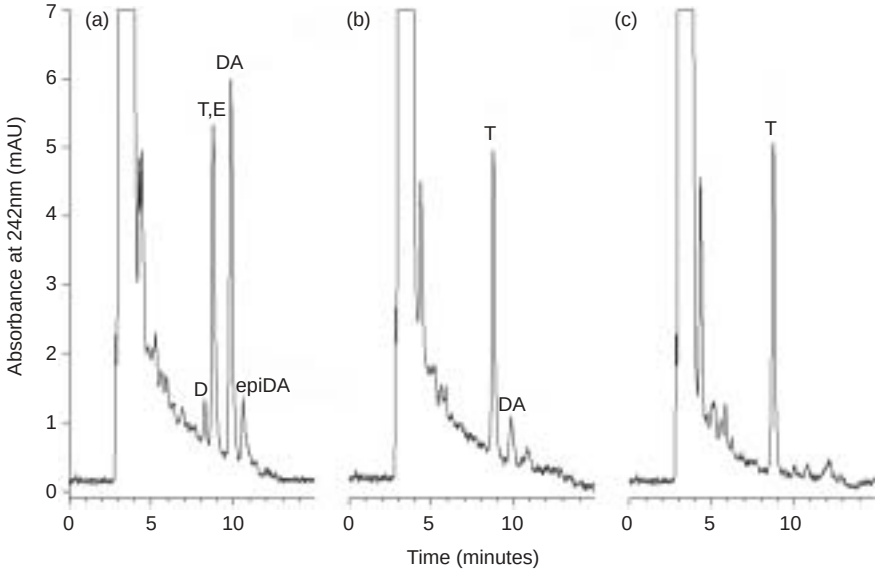

Figure 9.3
HPLC-UV chromatograms of the crude aqueous methanol extracts of three mussel tissues with A, 20 mg kg^{-1}; B, 2 mg kg^{-1}; C, 0 mg kg^{-1} domoic acid (produced by blending the MUS-1 reference material with control mussel tissues). Peak identities: DA, domoic acid; epiDA, 5'-epidomoic acid; D, E, isodomoic acids D and E (see Fig. 9.2); T, tryptophan. Conditions are reported in Appendix 9.2.

presents a detailed procedure for performing such analyses. Two other derivatization procedures for HPLC-FD have been developed recently. Sun and Wong (1999) developed a method based on the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate reagent for the analysis of domoic acid in plankton samples. James *et al*. (2000) utilized the 4-fluoro-7-nitro-2,1,3-benzoxadiazole reagent and reported a detection limit of 6 µg kg^{-1} in shellfish tissue.

9.3.5 Capillary electrophoresis

Capillary electrophoresis (CE) is a new technique that has tremendous potential for the analysis of marine toxins. This relatively simple method allows rapid, high-resolution separations of complex, polar compounds. A narrow bore (50–100 µm i.d.) fused-silica capillary tube filled with buffer is connected between two liquid reservoirs. After a small volume of sample (typically 1–10 nl) is injected into the capillary, a differential voltage of 20–30 kV is applied at the ends of the capillary. Ionic substances migrate as narrow bands down the capillary, eventually passing by a detector (UV absorbance, fluorescence, etc.). Two reports on the application of CE-UV to domoic acid have been published to date (Nguyen *et al*., 1990; Zhao *et al*., 1997), the latter with successful application to shellfish tissues based on the aqueous methanol extraction and SAX-SPE clean-up procedure. Detection limits are similar

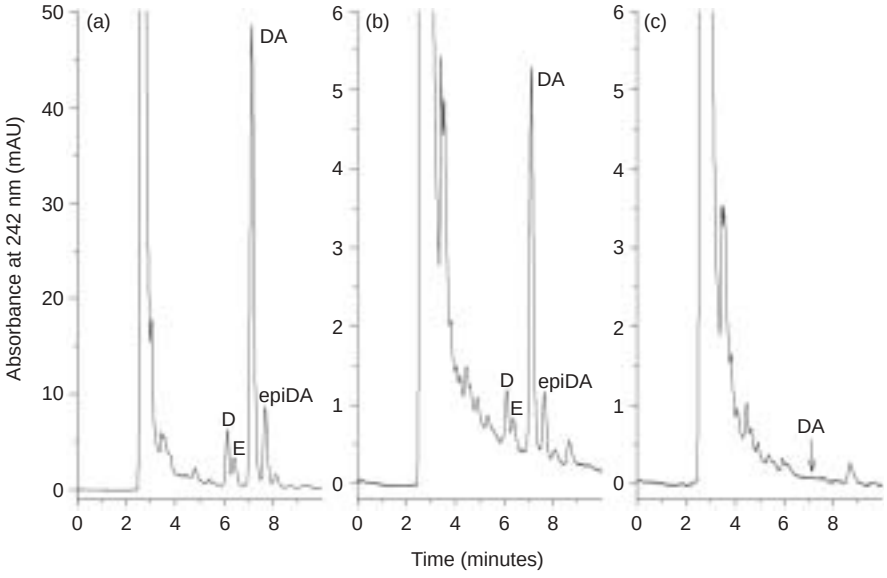

Figure 9.4
HPLC-UV chromatograms of the SAX-cleaned extracts of the same mussel tissues shown in Fig. 9.3, with A, 20 mg kg^{-1}; B, 2 mg kg^{-1}; C, 0 mg kg^{-1} domoic acid. Peak identities as in Fig. 9.3. Conditions are reported in Appendix 9.2. These analyses were performed on a different instrument than those in Fig. 9.3, which explains the different retention times.

to those of the HPLC-UV method. It has also been shown that the FMOC derivatization method can be applied to CE with fluorescence detection resulting in attomole (10^{-18} mol) detection limits (Quilliam *et al*., unpublished data).

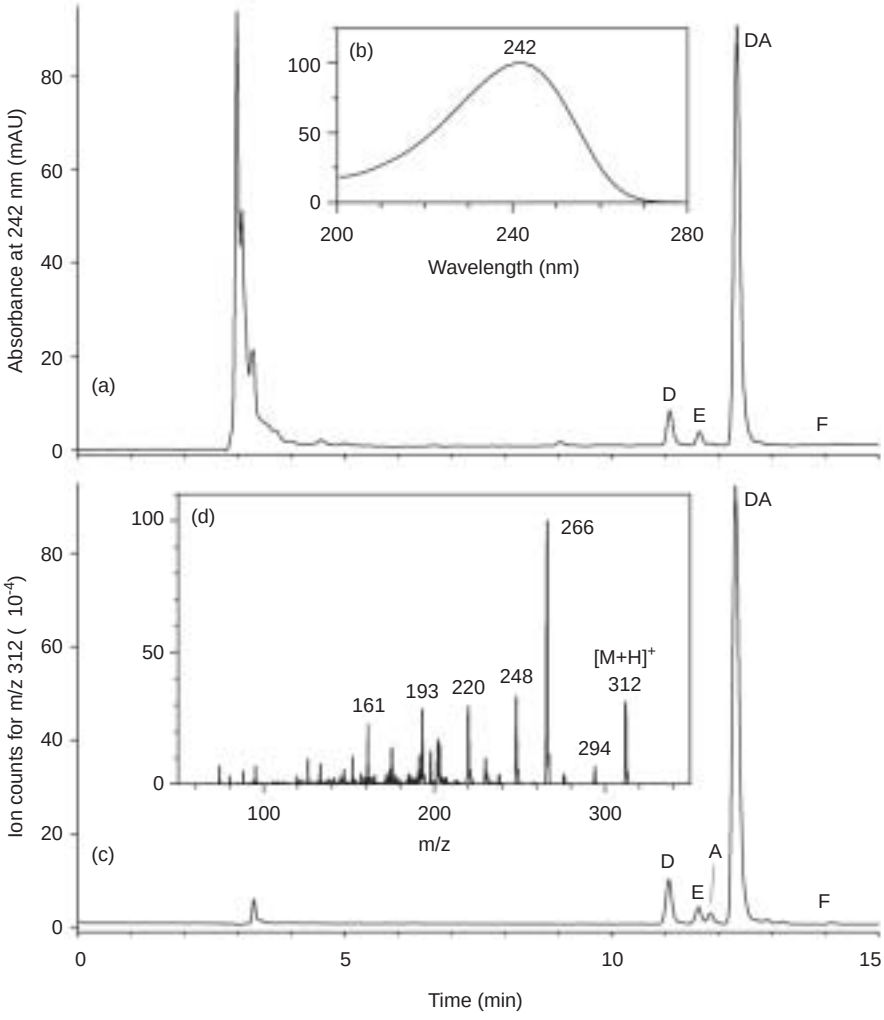

Figure 9.5
A, HPLC-UV and C, LC-MS chromatograms of the SAX-cleaned extracts of a razor clam sample contaminated with 80 mg kg^{-1} domoic acid. Insets B and D show respectively UV and MS-MS spectra of DA. Peak identities: DA, domoic acid; A, D, E, F, isodomoic acids (see Fig. 9.2). Conditions: gradient elution from 5% to 25% CH_3CN with 0.1% TFA, 4.6 × 250 mm Vydac 201TP column; the trace in C was acquired using selected ion monitoring, while the MS-MS spectrum in D was acquired by a separate run that scanned the fragment ions from collision-induced dissociation of mass: charge ratio (m/z) 312.

9.3.6 Mass spectrometry

Mass spectrometry is an accurate method for the quantitative and qualitative determination of domoic acid in a variety of matrices. For example, in early studies, fast atom bombardment (FAB) mass spectrometry using a direct probe inlet was found to be useful for the qualitative confirmation of domoic acid in concentrated HPLC isolates (Wright *et al*., 1989; Thibault *et al*., 1989). The development of an analytical procedure based on combined gas chromatography-mass spectrometry (GC-MS) for the analysis of domoic acid and related compounds in shellfish tissue samples has since been reported (Pleasance *et al*., 1990). Although this method is applicable to concentrations of domoic acid in contaminated shellfish ranging from 1–500 mg kg^{-1} wet tissue, its implementation required the development of an extensive clean-up procedure to facilitate derivatization to the N-trifluoroacetyl-O-silyl derivatives. Hadley and co-workers (1997) have developed an alternative procedure for GC-MS based on derivatization to the N-formyl-O-methyl derivative.

As HPLC-UV is the method most commonly used for routine regulatory purposes, a combined LC-MS method is most desirable for confirmation of peak identity. Various LC-MS interfaces for the analysis of ASP toxins have been investigated, including continuous-flow FAB, thermospray, atmospheric pressure chemical ionization and electrospray interfaces (Quilliam *et al*., unpublished data), and electrospray ionization was found to be best suited for analysis of domoic acid. In various communications (Quilliam *et al*., 1989*b*, 1996; Lawrence *et al*., 1994), electrospray LC-MS was shown to be a very promising method for the analysis of ASP toxins, as well as other marine toxins. LC-MS has a detection limit similar to that of HPLC-UV.

Fig. 9.5C shows the LC-MS analysis of a razor clam sample contaminated with domoic acid at 80 mg kg^{-1}. Very selective detection of domoic acid and its isomers is possible. Confirmation of identity can be achieved by scanning the mass spectrum with high front-end voltages that induce fragmentation or by performing MS-MS to generate a fragment ion spectrum such as that shown in the inset (Fig. 9.5D).

APPENDIX 9.1

Commercial sources of domoic acid

Pure domoic acid is commercially available through:

Diagnostic Chemicals Ltd, West Royalty Industrial Park, Charlottetown, PEI C1E 1B0, Canada; tel: +1 902-566-1396; fax: +1 902-566-2498; or DCL (USA), 160 Christian St, Oxford, CT 06478, USA; tel: +1 203-881-2020; fax: +1 203-888-1143; web: www.dclchem.com

Sigma Chemical Company, PO Box 14508, St Louis, MO 63178-9916, USA; tel: +1 800-325-3010, +1 314-771-5750; fax: +1 314-771-5757; web: http://www.sigma-aldrich.com

Certified Calibration Standards and Reference Materials

An accurate calibration standard and a certified mussel tissue reference material for domoic acid are available through the National Research Council (Canada) Certified Reference Materials Program (CRMP, Institute for Marine Biosciences,

1411 Oxford St, Halifax, NS B3H 3Z1, Canada; tel: +1 902-426-8280; fax: +1 902-426-5426; web: http://www.imb.nrc.ca/crmp_e.html; e-mail: crm.imb@nrc.ca). The mussel tissue reference material (Catalog No. MUS–1B, 4×8 g units) is certified to contain 36 µg domoic acid/g tissue and is stable for one year at 4ºC. This material is useful for testing the full implementation of an analytical method, such as the one detailed in Appendix 9.2, and for testing newly developed analytical methods (Hardstaff *et al*., 1990). The certified calibration solution (Catalog No. DACS–1C, 4×0.5 ml units) contains 100 µg/ml in aqueous 10% acetonitrile and is stable for one year at 4ºC in dark conditions.

APPENDIX 9.2

HPLC determination of domoic acid in shellfish tissues

This section presents a detailed procedure for the determination of domoic acid in shellfish (Quilliam *et al*., 1995). Domoic acid is extracted from shellfish tissues by homogenization with methanol-water (1:1, v/v). The concentration of domoic acid is determined by HPLC with UV absorbance detection. Sample extracts are injected following dilution and filtration of the crude extract or after clean-up on strong anion exchange (SAX) solid phase extraction (SPE) cartridges. The latter provides selective isolation of domoic acid and related compounds from interfering substances, as well as preconcentration to facilitate analysis of trace levels. The clean-up step is suitable only for unsalted product. Methodology for TLC analysis has been included for those laboratories not equipped with HPLC systems.

The procedures below can also be used to analyse plankton biomass isolated in the field by net tows or from cultures by centrifugation. However, the procedure presented in Appendix 9.3 has been designed specifically for samples of seawater containing plankton at low cell concentrations (100–1,000 cell ml^{-1}).

Reagents and equipment

(a) *Water:* distilled and passed through a water-purification system equipped with ion-exchange and carbon filters.
(b) *Solvents:* HPLC or distilled-in-glass grade acetonitrile and methanol, analytical grade formic acid, and spectrophotometric grade (>99% purity) trifluoroacetic acid (TFA).
(c) *HPLC mobile phase:* mix 100 ml acetonitrile with *c*. 400 ml water, add 1.0 ml TFA, and dilute to 1 l with water. Degas with ultrasonication and gentle vacuum.
(d) *Extraction solvent:* mix equal volumes of methanol and water.
(e) *Cartridge wash solution and injection diluent:* mix 1 volume acetonitrile with 9 volumes water.
(f) *Citric acid:* analytical grade citric acid monohydrate.
(g) *Tri-ammonium citrate:* analytical grade tri-ammonium citrate.
(h) *Citrate buffer eluent (0.5 M, pH 3.2 ± 0.2):* dissolve 40.4 g of citric acid monohydrate and 14.0 g of tri-ammonium citrate in 400 ml distilled water, add 50 ml acetonitrile and dilute to 500 ml with distilled water. (Tri-sodium citrate dihydrate (17.0 g) can be substituted for the ammonium salt. It is also possible to prepare this buffer by dissolving 52.55 g of citric acid monohydrate in 400 ml

distilled water, adjusting the pH to 3.2 with concentrated ammonium hydroxide (*c*. 13 ml), adding 50 ml acetonitrile and diluting to 500 ml with distilled water.

(i) *Domoic acid calibration solutions:* DACS–1C (certified 100 µg ml^{-1}, see Appendix 9.1) and accurate dilutions of DACS–1C in injection diluent (e) to give 1.0, 2.5, 10.0 and 25.0 µg ml^{-1}. Refrigerate solutions when not in use. Warm to room temperature before use. A small peak for the diastereomer, 5'-epi-domoic acid, is present in the HPLC trace of most standards. The compound separates from domoic acid on some columns but not others; the area for that peak should generally be included with that of domoic acid for purposes of calibration. The issue of whether to quantify the diastereomer in samples has not yet been addressed by regulatory authoritories.

(j) *Mussel tissue reference material:* MUS–1B (36 mg kg^{-1}, see Appendix 9.1).

(k) *Liquid chromatograph:* isocratic system equipped with UV detector capable of measuring absorbance at 242 nm and providing a S/N of 10:1 on injection of a 0.2 µg ml^{-1} domoic acid solution using conditions given below.

(l) *HPLC column:* 25 cm long × 4.6 mm i.d. packed with 5–10 µm C18 bonded silica gel (Vydac 201TP, Supelco LC-PAH, or equivalent); use of a guard column is recommended. Operating conditions: column temp. 40°C; mobile phase flow rate in the range 1.0–1.5 ml min^{-1}; injection volume 20 µl. If the HPLC is millibore compatible, a 25 cm long × 2.1 mm i.d. column can be substituted, using a 5 µl injection volume and mobile phase flow rate in the range 0.2–0.3 ml min^{-1}. Retention time of domoic acid should be 7–15 min (k' = 3 is best).

(m) *Strong anion exchange (SAX) cartridges:* 3 ml capacity, containing 500 mg of silica derivatized with a quaternary ammonium silane (JT Baker Scientific, Supelco, or equivalent).

(n) *Thin-layer chromatography plates:* activated silica gel 60 plates (Merck, F_{254}–250 µm).

(o) *TLC mobile phase:* Butanol-acetic acid-water (3:1:1, v/v/v).

(p) *TLC visualization reagent:* 0.30 g ninhydrin in 100 ml butanol-acetic acid (97:3).

Preparation of samples

1. *Tissue preparation:* After removal from the shell, drain tissues to remove salt water. For representative sampling it is advisable to homogenize 100 g of pooled tissue in a blender. Subsampling from this homogenate can be done immediately after blending while still well mixed, or later after mixing again. If an analysis must be performed on a limited amount of sample, a portion of chopped or ground tissue can be weighed directly into the extraction tube. Tissue homogenates may be stored for several weeks at -10°C or lower if tightly sealed.
2. *Extraction:* Accurately weigh 4.0 g tissue homogenate (as prepared in step 1) into a graduated centrifuge tube. Add 16.0 ml extraction solvent (d) (1:1 methanol-water) and homogenize the sample extensively (3 min at 3,000 × g). Do *not* try to recover all the tissue remaining in the blender or on the homogenizer probe but do wash it thoroughly afterwards to prevent contamination of the next sample. (Or, if a blender must be used, weigh the homogenate into a tared stainless-steel micro-blender cup, add 16.0 ml of extraction solvent (d)

and blend at medium speed for 4 min. Pour *c*. 15 ml of the resulting slurry into a centrifuge tube. The weight of tissue and volume of solvent may be scaled up if a micro-blender cup is not available.) Centrifuge at 3,000 × g or higher for 10 min. If analyses are not performed immediately, the extract may be stored in a tightly sealed screw-capped storage vessel in a freezer at *c*. -12°C. Prior to analysis or further processing, filter a portion of the supernatant through a dry methanol-compatible 0.45 µm filter.

3. *Diluted crude extract:* For screening samples for a high level of contamination and for salted samples, deliver 1.0 ml of filtered supernatant from step 2 into a 5 ml volumetric flask or graduated cylinder, dilute to 5.0 ml with water, mix, and analyse without the SAX cartridge clean-up (omitting steps 4 and 5).
4. *SAX cartridge conditioning:* First pass 6 ml of methanol, then 3 ml water, and finally 3 ml extraction solvent (d) through the SAX cartridges prior to use or testing. Do not allow the cartridges to go dry at any point in the procedure.
5. *SAX cartridge clean-up (for HPLC):* Load 5.0 ml filtered supernatant from step 2 on to the cartridge and allow it to flow slowly (about 1 drop per second). Stop flow just as the sample meniscus reaches the top of the cartridge packing. Discard the effluent. Wash the cartridge (at about 1 drop per second) with 5 ml of the cartridge wash solution (e). Stop the flow just as the solvent meniscus reaches the top of the cartridge packing. Discard the effluent. Add 0.5 ml citrate buffer eluent (h) and carefully allow it to flow just until the solvent meniscus reaches the top of the cartridge packing. Discard the effluent. Place a 2 ml volumetric tube or flask under the cartridge. Elute the domoic acid with 2 ml citrate buffer eluent (h) (slowly, 1 drop per second) just until the mark is reached on the volumetric tube. Mix the solution before withdrawing an aliquot for HPLC analysis. Samples should be analysed as soon as possible; otherwise store crude extracts tightly sealed in a screw-capped glass vial in the refrigerator (do not store for more than one week). The SAX-cleaned extracts should not be frozen, as domoic acid can decompose under such conditions.
6. *SAX cartridge clean-up (for TLC):* Use the same procedure as in step 5, except that the citrate buffer eluent (h) is replaced with 2% (v/v) formic acid in acetonitrile-water (1:9).

HPLC determination

Inject domoic acid calibration solutions (i) over the range of 1–100 µg ml^{-1}. If good linearity of response and a zero intercept are evident, single-point calibration (e.g. 10 µg ml^{-1}) may then be used routinely. Replicate injections should have a coefficient of variation (CV) <5%. Inject sample extracts (diluted and filtered crude methanol-water extracts from step 3 or SAX-cleaned extract from step 5 in 'Preparation of samples') in duplicate. Avoid carry-over between injections of different samples by washing the injector loop with injection diluent (e). Average peak areas for each sample. Repeat single injections of domoic acid calibration solution every 2 h and duplicate injections every 8 h. In calculations, average the peak areas of standards immediately following and preceding a series of samples.

Calculate the concentration of domoic acid (mg kg^{-1}) in each sample according to the following formula, where A_S is the average peak area for the sample, A_C is the average area for the calibration standard (concentration C_C µg ml^{-1}) bracketing the sample, W is the weight in grams of tissue homogenate extracted (*c*. 4.0 g), and F is

the dilution factor (F = 8 for the SAX-cleaned extract; F = 100 for the diluted crude methanol-water extract).

Concentration of domoic acid (mg kg^{-1} tissue) = $(A_S/A_C)(C_C/W)(F)$.

Thin-layer chromatography

Apply 50–100 µl of domoic acid calibration solution (i) or SAX-cleaned extract of mussel tissue or plankton from step 6 in 'Preparation of samples' to an activated silica-gel plate (Merck, silica gel 60 F_{254} 250 µm) and elute with butanol-acetic acid-water (3:1:1). After air-drying the plate, examine under short-wavelength UV light (254 nm). Domoic acid appears as a UV-quenching spot at approximately $R_f = 0.5$. After this, spray the dried plate (left 1 h in a fume hood) with a 1% solution of ninhydrin. Domoic acid appears as a yellow spot.

Blanks and recoveries

(a) *Extraction blank:* Perform step 2 in 'Preparation of samples', substituting 4 g water for the sample tissue. Analyse a portion of the diluted and filtered methanol-water extract from step 3, and then carry the remaining methanol-water extract through steps 4 and 5 and analyse again. Chromatograms should be free of peaks eluting near domoic acid or causing excessive baseline slope. As needed, replace methanol, water, modify between-sample rinsing procedures, use alternative brand of SAX column, etc.

(b) *SAX-column recoveries:* Perform an HPLC determination in duplicate of a filtered solution containing domoic acid in extraction solvent (d) in the range 10–30 µg ml^{-1} (ideally this should be an extract prepared from the tissue of interest, either naturally contaminated or spiked with domoic acid or a domoic acid-containing tissue). Using three cartridges from the lot to be used, take the same solution through the SAX clean-up (steps (4) and (5) in 'Preparation of samples'). Perform the determination in duplicate for each of the three eluates. Calculate the percentage recovery for each eluate using the appropriate dilution factors and the average domoic acid level determined in the crude extract. All three recoveries determined should fall in the range 85–115%, with an average recovery >90%. If the recoveries determined do not satisfy these criteria, try another source of SAX cartridges.

APPENDIX 9.3

HPLC determination of domoic acid in seawater and plankton

This section presents a detailed procedure for the determination of domoic acid in seawater and plankton samples (Pocklington *et al*., 1990). The method involves pre-column derivatization with 9-fluorenylmethylchloroformate to form the FMOC derivative followed by reversed-phase HPLC with fluorescence detection. The detection limit for domoic acid in seawater with an isocratic HPLC system is about 0.5 ng ml^{-1}, while a gradient elution system can detect as low as 15 pg ml^{-1}. These detection limits permit the analysis of *Pseudo-nitzschia* culture samples with cell densities in the range 1,000–10,000 cells ml^{-1} or 20–10,000 cells ml^{-1}, respectively, assuming domoic acid at 1–20 pg $cell^{-1}$.

Reagents and equipment

(a) *Water:* distilled and passed through a water-purification system equipped with ion-exchange and carbon filters.
(b) *Seawater:* filtered through a glass-fibre filter (Type A/E, Gelman Sciences Inc., Ann Arbor, Mich.) prior to use.
(c) *Solvents:* HPLC or distilled-in-glass grade acetonitrile, HPLC-grade ethyl acetate, and spectrophotometric grade (>99% purity) trifluoroacetic acid (TFA).
(d) *9-fluorenylmethylchloroformate (FMOC-Cl):* available from Aldrich Chemical Co. (Milwaukee, Wis.).
(e) *FMOC-Cl reagent solution (15 mM):* dissolve 38.7 mg FMOC-Cl in 10.0 ml acetonitrile. Store in 2 ml glass screw-cap vials with Teflon-lined septa in a dessicator at -20°C. All vials should be purged with nitrogen prior to storage. Once the septum has been pierced, any unused reagent should be discarded.
(f) *Borate buffer (1M, pH 6.2):* dissolve 6.18 g orthoboric acid (BH_3O_3) in 95 ml deionized water, adjust the pH to 6.2 with 2 M sodium hydroxide, and dilute with water to 100 ml.
(g) *Domoic acid calibration solutions:* DACS–1C (certified 100 µg ml^{-1}, see Appendix 9.1). Prepare working solutions daily by accurately diluting DACS–1C in seawater to give 5, 10, 25, 50, 100, 200 and 500 ng ml^{-1}.
(h) *Dihydrokainic acid(DHKA):* available from Sigma Chemical Company (St Louis, Mo.)
(i) *DHKA internal standard solution:* prepare a stock solution (100 µg ml^{-1}) by dissolving 1.0 mg DHKA in 10.0 ml acetonitrile-water (1:9). Prepare a working solution (2 µg ml^{-1}) by diluting the stock solution 50-fold in seawater.
(j) *HPLC mobile phase: (i) isocratic system:* 40% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid in water. *(ii) gradient system:* solvent A = 0.1% TFA in water; B = 0.1% TFA in acetonitrile. Degas with ultrasonication and gentle vacuum.
(k) *Liquid chromatograph:* isocratic or preferably gradient system equipped with a dual monochromator fluorescence detector set for excitation at 264 nm and emission at 313 nm protected by a 280 nm cut-off filter. A filter fluorometer equipped with the appropriate filters may be substituted.
(l) *HPLC column:* 25 cm long × 4.6 mm i.d. packed with 5 µm C18 bonded-silica gel (Vydac 201TP, Supelco LC-PAH, or equivalent); use of a guard column is recommended. Operating conditions: ambient column temp. for isocratic, 55°C for gradient; 1.0 ml min^{-1} mobile phase flow rate; 10 µl injection volume for isocratic, up to 100 µl for gradient. If the HPLC is millibore compatible, a 25 cm long × 2.1 mm i.d. column can be substituted, using a 2 µl injection volume for isocratic or 20 µl for gradient, and a mobile-phase flow rate of 0.2 ml min^{-1}. Gradient elution: programmed linearly from 30–50% B over 15 min, followed by an increase to 100% B over 2 min which is maintained for 5 min before programming back to initial conditions over 2 min. Maintain initial conditions for a further 12 min before the next injection.

Preparation of samples

1. For determination of domoic acid dissolved in seawater or culture media, filter a 1 ml subsample through a 0.22 µm disposable filter (Millex-GS, Millipore Corp., Bedford, MA). Take the filtrate through the following derivatization and HPLC procedures.

2. For determination of overall concentrations of domoic acid in plankton culture samples or suspensions of plankton in seawater, first determine the cell density in representative subsamples. Sonicate a homogenous subsample (10 ml) for 1 min at 100 W using a 1 cm diameter probe to disrupt the cells. Then filter 1 ml of sample through a Millex-GS 0.22 µm disposable filter. Take the filtrate through the following derivatization and HPLC procedures.
3. For determination of concentrations of domoic acid in phytoplankton cells as well as in the medium, first determine the cell density in representative subsamples. Centrifuge a 10 ml aliquot of sample in a conical tube for 5 min at approximately 900 × g. Remove 5 ml of supernatant and treat as in step 1. Centrifuge the remaining material for another 5 min at 900 × g. Remove all but approximately 0.2 ml supernatant, make up the volume to 10 ml with seawater. Sonicate for 1 min at 100 W using a 1 cm diameter probe to disrupt the cells. Then filter the solution through a Millex-GS 0.22 mm disposable filter. Take the filtrate through the following derivatization and HPLC procedures.
4. For other samples, such as plankton biomass (fresh or freeze-dried) or plankton cells isolated on filters, first suspend the cells in seawater and then proceed with step 3.

Derivatization

Mix the following in a glass test tube (10 mm × 75 mm)using a vortex mixer for 10 s: 200 µl sample, calibration solution (g) or seawater blank; 50 µl borate buffer (f); and 10 µl DHKA internal standard solution (i). Then add 250 µl FMOC-Cl reagent solution (e) and mix. After exactly 45 s, add 500 µl ethyl acetate and mix for an additional 45 s. After the mixture has settled, two distinct phases will be visible. Remove the upper organic layer with a disposable glass pipette and discard. This is repeated with two more 500 µl portions of ethyl acetate and 20 s mixing. Transfer most of the aqueous bottom layer to a vial for HPLC analysis.

The control of contamination is important when working with samples having low domoic acid concentrations. All glassware, syringes and vials should be rigorously washed (water, methanol, acetone). Deliver all reagents using glass syringes dedicated to each solution, and deliver the sample with an air displacement pipette with disposable tips.

HPLC analysis

Inject derivatized calibration solutions (g) over the concentration range 10–300 ng ml^{-1}. If good linearity of response and a zero intercept are evident, single-point calibration may then be used routinely. Replicate injections should have a CV <5%. Inject derivatized blank seawater samples. There should be no interference at the retention time for domoic acid. Avoid carry-over between injections of different samples by washing the injector loop with acetonitrile and water. Inject derivatized sample extracts. Repeat single injections of calibration solution every 2 hours. In calculations, average the peak areas of standards immediately following and preceding a series of samples.

Calculate the concentration of domoic acid (ng ml^{-1}) in each sample according to the following formula, where A_S and I_S are the average peak areas of domoic acid and internal standard (DHKA) for the sample, respectively, and A_C and I_C are the

corresponding average peak areas for the calibration standard of concentration C_C (ng ml^{-1}) bracketing the sample.

Concentration of domoic acid (ng ml^{-1}) = $(A_S/A_C)(I_C/I_S)(C_C)$.

Calculate the concentration in plankton cells (pg cell^{-1}) according to the following formula, where D is the cell concentration (cells ml^{-1}).

Concentration of domoic acid (pg cell^{-1}) = $(1{,}000)(A_S/A_C)(I_C/I_S)(C_C/D)$.

Safety

Domoic acid is a neurotoxin and must be handled with caution. Acetonitrile and methanol are toxic, volatile solvents. Trifluoroacetic and formic acids are toxic, volatile and corrosive and should only be handled in a fume hood. As for 9-fluorenylmethylchloroformate, it is toxic and a skin irritant. All these substances are harmful if swallowed, inhaled or absorbed through the skin.

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10

Detection of toxins associated with ciguatera fish poisoning

R. J. Lewis

10.1 INTRODUCTION

Ciguatera is a disease caused by heat-stable sodium-channel activating toxins that cause a wide array of gastrointestinal and neurological symptoms (Gillespie *et al*., 1986; Lewis and Ruff, 1993; Lewis *et al*., 2000). This disease follows consumption of fishes that have accumulated a number of ciguatoxins (CTXs) derived from the benthic dinoflagellate, *Gambierdiscus toxicus* through the marine food chain (Fig. 10.1; Lewis and Holmes, 1993). The most potent sodium-channel toxin known, P-CTX-1, is the major toxin found in carnivorous fish, where it typically contributes about 90% of total lethality and poses a health risk at levels >0.1 ppb (μg kg^{-1}) (Murata *et al.*, 1990; Lewis *et al.*, 1991; Lewis and Sellin, 1992). Ciguatera can be debilitating and slowly resolving but is seldom fatal. In societies that depend heavily on reef fish for protein (e.g. atoll island countries of the Pacific), ciguatera can have major adverse socioeconomic impacts, whereas in Western societies outbreaks of ciguatera often attract media attention with a consequent negative effect on the marketing of seafood (Lewis, 1992, 1994). Victims of ciguatera may seek compensation through the courts (Payne, 1994).

Development of a cost-effective means of detecting ciguateric fish prior to consumption remains one of the few management options that can directly reduce the adverse effects of ciguatera. Antibody-based assays and sodium-channel binding assays appear to hold the most promise because they can detect, under favourable circumstances, targeted compounds at picomolar levels and can be developed as cost-effective screens (Gazzaz *et al*., 1992). For example, Gathumbi *et al*. (2001) recently produced a rabbit polyclonal antibody-based enzyme immunoassay (EIA) with a detection limit of 16 pg ml^{-1} for aflatoxins. However, until high-affinity antibodies to the CTXs are produced, the mouse bioassay remains the principle method of detection for many laboratories, despite attendant concerns for animal welfare.

In this chapter, details are given on the mouse bioassay for estimating ciguatoxicity in fish and discussion is provided on progress towards the development of alternative *in vivo* and *in vitro* assays for detecting ciguateric fish. Chapter 8 should be consulted for the application of chemical analytical methods for lipophilic toxins, such as CTXs, and details on alternative assays are found in Chapters 12 and 13. Background on the immunological, biochemical and chemical features of the CTXs relevant to their differential detection has been published (Lewis, 1995).

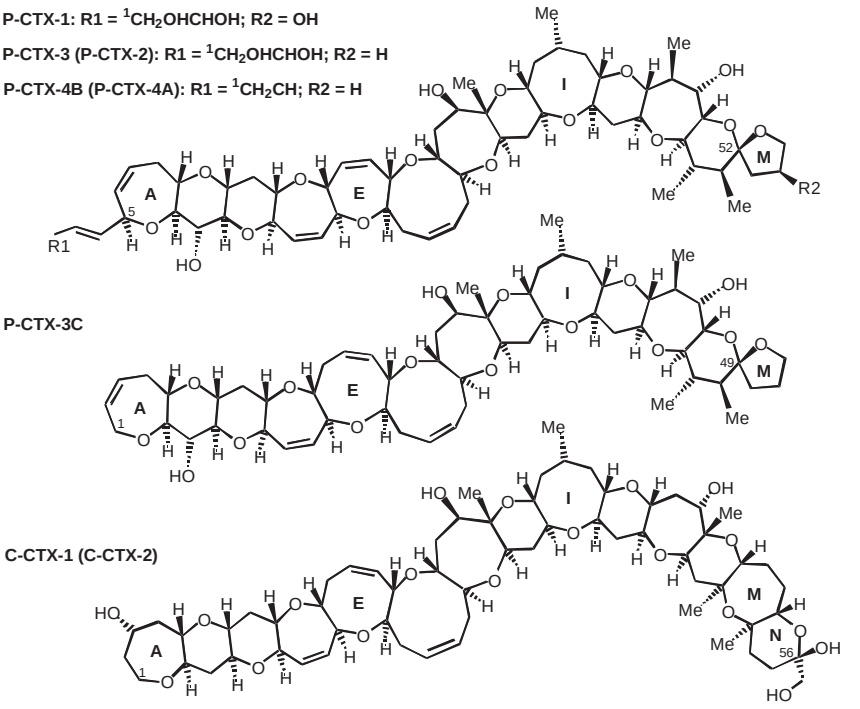

Figure 10.1
Structures of the major ciguatoxins (CTX) present in the flesh of ciguateric fish in the Pacific and Caribbean. Epimers at carbon positions C52, C49 or C56 are named in parentheses.

10.2 In vitro bioassays

10.2.1 Antibody assays

The potential of antibody-based screening assays to directly detect CTX in fish flesh was first indicated by Hokama *et al.* (1977). Improvements have been made on the format and antibodies used in the test (Hokama, 1985; Hokama *et al*., 1983, 1985, 1989), which led to the assay being sold by Hawaii Chemtec International. Independent evaluation of the test (Dickey *et al*., 1994) indicated it gave less than satisfactory results, and the test was not a commercial success. Oceanit of Hawaii has now produced a second commercial test based on Hokama's antibodies and in a modified test format (Hokama *et al*., 1998). More than 25,000 Cigua-Check® tests have been sold, with no confirmed reports of false negatives (http://www.cigua.com). Cigua-Check® detects CTX at concentrations that cause clinical symptoms (>0.08 µg kg^{-1}), whereas certain other marine polyether toxins (e.g. okadaic acid, brevetoxins) are detected in fish flesh at levels approximately 5–10 times higher. With a claimed reliability of nearly 100% and a specificity of 86%, Cigua-Check® is currently undergoing an Association of Official Analytical Chemists collaborative study. The

test currently sells for less than US$6 per assay; Oceanit Test Systems also provides their own testing service (Cigua-Check® assay [US$50], crude extraction [US$150], bioassay [US$100] and neuroblastoma assay [US$150]).

Recently, antibodies raised to a synthetic JKLM ring fragment of CTX were found to detect CTX-1 at levels of 1.2×10^{-9} M using a biotin-avidin amplification system (Pauillac *et al*., 2000). This antiserum did not significantly cross-react with a brevetoxin congener (PbTx-3), okadaic acid, monensin or other polyether compounds. It remains to be determined if the antibodies produced by this approach have sufficient affinity and selectivity for the detection of CTXs in fish. In addition to the major CTXs, low-potency CTXs arising from further *in vivo* biotransformation may also accumulate in fish to appreciable levels (Lewis and Holmes, 1993). While the low potency of these CTXs has made their detection difficult by the mouse bioassay, such compounds may cross-react with antibodies to CTX-1, to increase the probability of obtaining false-positive results. Unfortunately, antibody-based assays respond depending on the relative affinity (specificity) of the antibody for each form of the toxin in a way that is not necessarily related to the potency of the different forms.

10.2.2 Sodium-channel binding assays for ciguatoxins

The CTXs are characterized by their high affinity, quasi-irreversible binding with an effective dose (expressed as ED_{50}) <0.2 ng ml^{-1} to voltage-sensitive sodium channels (Lewis *et al*., 1991). Importantly, the affinity of each CTX for the sodium channel is proportional to its intraperitoneal (i.p.) LD_{50} in mice (Lewis *et al.*, 1991). In contrast to antibody-based assays, sodium-channel-based assays can provide a direct measure of *in vivo* potency (see also Chapters 12 and 13). The CTXs bind to site 5 on sodium channels, a site overlapping the brevetoxin binding site (Lombet *et al.*, 1987) and can be detected by measuring the inhibition of the binding of [^{3}H]-brevetoxin to sodium channels. This assay has been used to help to isolate new CTXs (Poli *et al*., 1997). Ciguatoxin binding to sodium channels causes them to open at normal cell-resting membrane potentials (Lewis *et al*., 2000). In intact cells, this results in the influx of sodium ions, cell depolarization, and the appearance of spontaneous action potentials in nerves. This sodium influx can be markedly enhanced by the addition of site 2 sodium-channel activators such as veratridine through an allosteric mechanism. The cell-based assay for the CTXs (Manger *et al*., 1993, 1994*a*, 1994*b*) (see Chapter 12) takes advantage of this phenomenon, and this assay is highly sensitive to CTXs and other site 5 sodium-channel activator toxins. A reporter gene cell-based assay has now been developed for marine algal toxins including CTXs (Fairey and Ramsdell, 1999). These sodium-channel assays have the potential to be automated and miniaturized but require somewhat specialized laboratory equipment. The *in vitro* sodium-channel assays described above are more sensitive than the mouse bioassay and have considerable potential to replace this assay for the detection of CTXs in crude fish extracts. However, the nature and extent of clean-up losses when extracting CTX from fish flesh have not been well characterized, and care must be exercised to ensure that the lipids present in crude extracts of fish do not give rise to false-positive results.

10.3 *IN VIVO* BIOASSAYS FOR CIGUATOXINS

The mouse bioassay, based on the method described by Banner *et al*. (1960, 1961), is currently the most widely used assay for the detection of CTXs in contaminated fish. This assay has recently been validated for the detection of CTXs in up to 20 mg of ether extract of fish flesh (Lewis and Sellin, 1993). Step by step procedures for the extraction and partial purification of CTXs from fish tissues and the bioassay of these extracts using mice are herein described in detail.

10.3.1 Extraction of ciguatoxins from fish tissue

A flow diagram of the steps involved in the extraction of CTXs for mouse bioassay is shown in Fig. 10.2. Samples should be in good condition upon collection, clearly labelled and immediately frozen at <-20°C for storage. Prior to extraction, samples are thawed (store >50 g of flesh for testing with additional assays), cooked in a plastic bag resistant to boiling, chopped and/or minced to a fine slurry, and either refrozen prior to extraction or extracted immediately after the sample has cooled to room temperature. The cooking step (which could be done in a microwave oven) is included to denature proteins that can interfere with the physical process of homogenization in acetone and reduce extraction efficiency. Ciguatoxins are extracted with acetone, along with a wide range of other lipid soluble material. Typically, samples are extracted by homogenization for 15 min using an explosion-proof homogenizer, such as an air-powered model fitted with a medium-viscosity generator with protruding cutters. Blending for this length of time will result in high temperatures, care should be taken to monitor and control the temperature, to ideally <80°C. Samples are extracted twice with acetone at room temperature and the acetone-soluble components are separated from insoluble material by vacuum filtration through filter paper in a Buchner funnel. As the first extract is composed of approximately 20% water, a large quantity of interfering water-soluble compounds is co-extracted. A separate resuspension in fresh acetone followed by filtering is required. The extract is then dried to a viscous slurry on a rotary evaporator. More rapid drying on the rotary evaporator may be achieved by keeping the first and second extracts separate until almost dry. To ensure that samples do not foam excessively, small amounts of antifoaming agent (e.g. n-octanol) may be added to difficult samples. A tap placed in the vacuum line entering the rotary evaporator can be used to maintain the vacuum at a suitable pressure and reduces the risk of excessive foaming and 'bumping'. The subsequent liquid-liquid partitioning steps are described in detail in Fig. 10.2.

Care must be taken to ensure that pressure in the separatory funnel is regularly released during shaking and that the correct layers are carefully collected. With this procedure about 63% of CTX is recovered from fish flesh using routine methods (Lewis and Sellin, 1993). Unfortunately, in the mouse assay, 20 mg of an ether extract is typically insufficient to detect the presence of CTXs in low-toxicity ciguateric fish (Lewis and Sellin, 1993). Several modifications to this approach have been described. Replacing the ethanol-water/ether partition with a 2 M NaCl/ether partition may yield a two-fold improvement in sample clean-up (Lewis and Sellin, 1993). A truly rapid, inexpensive extraction procedure for the CTXs has not yet been developed. The extraction of toxins from benthic dinoflagellates requires additional care because of the presence of other toxins in addition to those found in the tissues of ciguateric fish (Lewis, 1995).

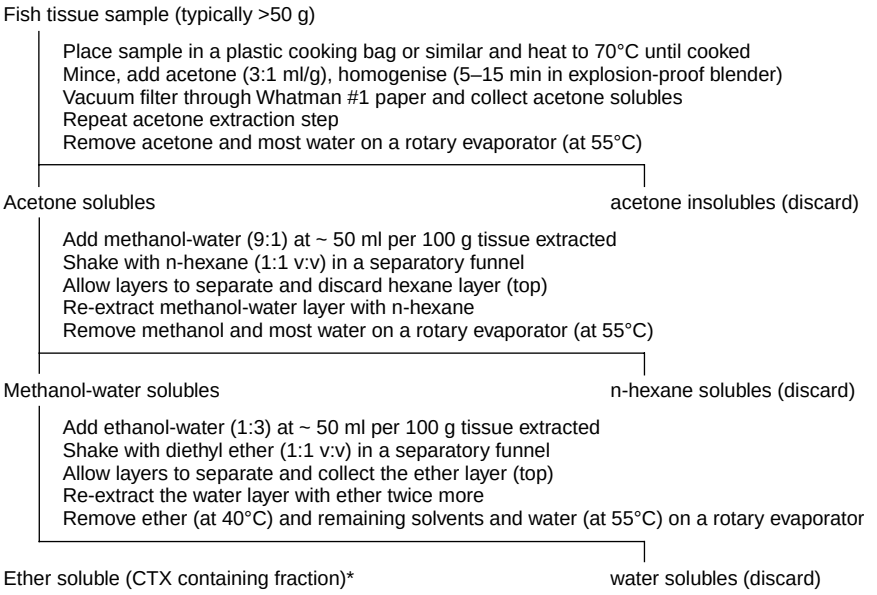

Figure 10.2
Generalized scheme for the extraction and partial purification of the CTXs from fish tissue. The ether soluble material* contains the CTXs (and any gambiertoxins) and is suitable for testing by the mouse bioassay and ready for further purification. Fractions required for testing should be stored at -20°C, either dry under N_2 or dried and then made up to a known volume with chloroform–methanol (97:3). Discarded can be dried and assayed if it is suspected that toxins are present with polarity (solubility) different from the CTXs and gambiertoxins. The water-bath temperatures indicate work on a rotary evaporator with tap-water cooling and a weak vacuum system (e.g. water aspirator). Lower bath temperatures and higher vacuums can be used with colder traps (e.g. dry ice/ethanol or dry ice/acetone). A tap placed in the vacuum line before the rotary evaporator can be used to easily maintain vacuum at the correct level. This constitutes a closed system, ensuring that solvents do not enter vacuum pumps or waste-water outflows. Chloroform–methanol (9:1) can be used to transfer samples from large flasks (used to dry large or difficult samples, especially acetone-water, methanol-water or water containing fractions) to smaller flasks for final drying and sample weighing. Both partitioning solvents should be used to transfer samples efficiently from rotary evaporator flasks to the separatory funnel.

10.3.2 Mouse bioassay

A step by step description of the mouse bioassay for CTXs is given in Table 10.1. Briefly, a portion (up to 20 mg per mouse) of each diethyl ether fraction is suspended in 0.5 ml 1–5% Tween 60/0.9% saline and assayed in duplicate by i.p. injection into healthy mice of either sex weighing 20 g ± 2 g. Control mice

receive Tween/saline alone. For each mouse, weight, sex, time of injection, quantity injected, signs of intoxication, rectal body temperature (optional) and the time to death are recorded. Mice should be observed and monitored closely for the first two hours, then intermittently over at least 24 h, with mice close to death being monitored closely. A depression of growth at four days can also be used to indicate the presence of sub-lethal doses of CTX (Lewis *et al*., 1993). The relationship between dose and time to death is used to quantify each fraction. For the mix of CTXs typically found in carnivorous fish of the Pacific (Lewis and Sellin, 1992) this relationship is approximated by: $-\log MU = 2.3 \log(1 + T^{-1})$, where MU = number of mouse units of CTX injected and T = time to death in hours. One MU is the lethal dose (LD) for a 20 g mouse, which is equivalent to 5 ng CTX-1 (Lewis *et al*., 1991). The mouse assay of ether extracts of fish tissue is considered to be a reliable method of characterizing and quantifying the presence of CTX. However, reliable characterization requires that the signs of intoxication in mice are consistent with CTX, especially the presence of hypothermia below 33°C, dyspnoea with gasping, severe diarrhoea or lachrymation or hypersalivation, preferably with an estimate (derived from testing several doses) that the dose time to death relationship approximates the relationship for CTX (Lewis and Sellin, 1993). Additional signs of mouse intoxication have been reported by Hoffman *et al*. (1983) for a partially purified extract from a Caribbean fish; however, some of these additional signs are not easily observed and are often absent when Pacific CTXs are tested.

It is important to note that i.p. administration of 20 mg of crude ether extract from non-toxic fish can induce signs of mild intoxication in mice that could be misinterpreted (Lewis and Sellin, 1993). For accurate quantification of dose, a specific dose time to death relationship should be established using mice from the locally available breeding colony, preferably for each source of CTX to be assayed. The strain and housing conditions for mice can influence sensitivity to CTX, in addition to any differences caused by the presence of different toxins or different ratios of the same toxins (Lewis, unpublished observations). A dose time to death relationship is obtained by injecting about 12 mice (19–21 g) with doses between 1 MU and 20 MU of toxin.

The use of the appropriate dose time to death relationship allows the accurate quantification of a particular toxin or mix of toxins with a minimum number of mice (typically only two mice are required) and can quantify unknown potency extracts over a wide range of doses (1–20 MU) with acceptable accuracy, if doses giving death times <40 min are retested at lower doses. This approach to estimating the LD_{50} of a fraction avoids a number of problems associated with classical LD_{50} estimations, where additional mice are required for initial range-finding, and many more mice receive close to the minimum lethal dose and consequently are affected longer by the toxin. Given that putatively toxin-free extracts at 1 g kg^{-1} (dose weight per mouse body weight) can induce signs of intoxication following i.p. injection into mice (Lewis and Sellin, 1993), the presence of specific toxin(s) in crude extracts given at i.p. doses above 1 g kg^{-1} requires independent confirmation. The presence of toxins likely to be involved in ciguatera should be questioned if such doses are not orally equipotent – the CTXs are similarly potent whether administered i.p. or orally to mice (Lewis *et al*., 1991). It is noteworthy that free unsaturated fatty acids from marine species have an i.p. LD as low as 300 mg kg^{-1} (Takagi *et al*., 1984).

TABLE 10.1 Mouse assay to detect CTX in extracts from suspect ciguateric fish

Preparation of ether soluble extract for injection

- Dissolve ether extract in chloroform–methanol (97:3) up to a known volume; remove an aliquot containing the required weight of extract to be assayed[a]
- Remove solvent from the sample on a rotary evaporator and/or under a stream of N_2
- To the dried material, add 1–5% Tween 60/0.9% saline solution to give a final volume of 0.1–0.5 ml for each intraperitoneal (i.p.) injection into a mouse
- Warm, whirl-mix and/or sonicate to ensure extract dissolves or is evenly suspended prior to any transfers (use 5% Tween 60 saline to suspend difficult samples)

Mouse assay

- Obtain healthy 18–22 g mice of either sex,[b] house at ~25°C (12:12 light–dark), and provide food and water *ad libitum*
- Mark mice for easy identification and place them in paper-lined cages (do not reuse mice)
- Withdraw required volume of the Tween suspension (0.1–0.5 ml) into a 1 ml insulin syringe or similar fitted with a fine short needle
- In the first instance, inject ≤20 mg of extract into mice via the i.p. route
- For each mouse record: time of injection, weight, sex, extract (g) administered, time of onset and nature of signs, time of death (observe mice for at least 24 hours)
- Use two mice fraction per sample per dose, retest at a lower estimated dose (~2 MU) when deaths occur within 40 min (i.e. dose exceeds 10 MU of CTX)
- For extracts inducing hypothermia to <33°C, dyspnoea and gasping, as well as copious diarrhoea, lachrymation and/or hypersalivation, calculate MU injected from the time to death versus dose relationship for mixed CTXs[c]
- Non-lethal fractions at 20 mg inducing the above signs and poor weight gain at four days are estimated to contain 0.5 MU of CTX
- Fractions inducing unexpected signs or atypical dose time to death relationships could be retested to assess oral potency, as CTXs are equipotent by the i.p. and oral route

a. Slightly polar solvents (e.g. chloroform–methanol 1:1) may improve solubility of ether extracts, while even more polar solvents (e.g. methanol–water 9:1) may improve solubility of water and butanol soluble extracts.
b. Quackenbush mice can be bred by continuously keeping mating pairs together, reducing each litter to 10 around day two, and weaning mice at 21 days. Breeding females can be kept for 6 months and males for 12 months. A sheltered, low-noise environment is important for a successful breeding colony.
c. For the mixed CTXs found in carnivorous fish, log MU = 2.3 log$(1 + T^{-1})$, where one MU = 5 ng P-CTX–1 and time to death (T) is measured in hours to the nearest minute (Lewis and Endean, 1984). For routine assay of CTXs, a dose versus time to death relationship should be established for each colony of mice and for fish from different trophic levels and regions.

10.3.3 Alternative *in vivo* bioassays

A number of other animal assays have been reported for the detection of ciguatoxicity. These bioassays are in use in only a few laboratories and each has its strengths and weaknesses. The relative merits of the chicken, cat, mongoose, brine shrimp, mosquito and Diptera (fly) larvae assays are briefly discussed below. The chicken assay developed by Vernoux *et al.* (1985) provides a rapid means of assaying the toxicity of fish liver by administering minced portions of liver directly into the crop of young chickens at 10% of the body weight. Administration of flesh is physically more difficult but can be accomplished on most fresh samples. The mongoose

(Banner *et al*., 1960) and cat (Hessel *et al*., 1960; Bagnis *et al*., 1985; Lewis, 1987) can also directly assay the whole flesh of fish without the need for extraction but require considerably more sample for assay (5–15% fed) than the chicken. The chicken, mongoose and cat assays are semi-quantitative, sensitive and CTX induces characteristic signs of intoxication. However, these test species are typically not available through laboratory animal suppliers or are costly and results may be compromised because of disease. The continued use of large animal assays for detection of the CTXs is increasingly difficult to justify on ethical grounds.

The first non-vertebrate assay developed for detecting CTXs was the brine shrimp assay (Granade *et al*., 1976). However, attempts to confirm the usefulness of this assay by testing ether extracts from highly toxic ciguateric fish from Queensland were unsuccessful (Lewis, unpublished data, 1980). False-positive results were caused by the toxic effects on brine shrimp of the Tween 60 recommended to emulsify the extract and no toxic effect attributable to CTX could be detected. The mosquito bioassay has been extensively used in French Polynesia to assay levels of CTX in fish implicated in human poisoning (Bagnis *et al*., 1985, 1987). This bioassay correlates well with the cat, mouse and human responses. However, few laboratories perform this assay, perhaps because of difficulties obtaining and housing mosquitoes and a lack of familiarity in handling and recognition of signs characteristic of intoxication by CTXs. Recently, Labrousse and Matile (1996) proposed a dipterid larvae assay for detecting CTXs. The assay involves the direct feeding of suspect fish flesh to meat-eating fly larvae, with reduced weight gain (compared with controls) indicating that CTX is present. While the assay is inexpensive, rapid and relatively easy to establish with limited laboratory resources, further studies are required to improve sensitivity for Pacific and Indian Ocean CTXs (Lewis, unpublished data, 1995) .

10.4 Chemical methods for detecting ciguatoxins

10.4.1 Chemical features of ciguatoxins

The CTXs (Fig. 10.1) are lipid-soluble toxins consisting of 13–14 rings fused by ether linkages into a mostly rigid, ladder-like structure (Murata *et al*., 1990; Lewis *et al*., 1998). They are relatively inert molecules which remain toxic after cooking and exposure to mild acidic and basic conditions. In the Pacific, P-CTX-1 is the major toxin (on the basis of both quantity and total toxicity) present in fish, except certain herbivorous species that accumulate mostly gambiertoxins and less polar CTXs. An assay selective for P-CTX-1 would bensetul for the detection of ciguateric fish in the Pacific. In the Caribbean, C-CTX-1 appears to be the major toxin in many ciguateric species (Lewis *et al*., 1999).

10.4.2 Mass spectrometry detection of ciguatoxins

It is not possible to develop a method for selectively detecting CTX from other lipids present in a crude lipid extract from fish by monitoring the liquid chromatography (LC) eluant with a UV detector because CTXs do not possess a distinctive UV chromophore. However, CTXs are readily detected as sodium or ammonium adducts in positive-ion mode mass spectrometry (MS) (see also Chapter 8). Using LC/MS (Lewis and Jones, 1997; Vernoux and Lewis, 1997) and LC/MS/MS (Lewis *et al*.,

1999), nanogram quantities of CTXs present in partially purified extracts can be identified and quantified. Such an analytical detection methodology could be used to confirm CTX contamination and to validate positive (or negative) responses obtained by antibody-based assays. Preliminary studies with CTX-1 indicate that such an approach could form the basis of a confirmatory analytical assay for CTXs in fish (Lewis *et al*., 1999). However, simple extraction and clean-up protocols have not yet been developed to simplify this procedure. Such extraction procedures need to be tailored for a specific assay. There is considerable scope to develop improved rapid extraction methods that minimize interfering matrix effects.

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11

Marine cyanobacterial toxins

B. M. Long and W. W. Carmichael

11.1 Introduction

While many marine toxins have been identified from eukaryotic algae, the prokaryotic marine cyanobacteria have become a major source of bioactive and toxic metabolites from the marine environment. Several species and strains of cyanobacteria cause an often acute and potentially fatal condition in humans and other animals from drinking water that contains high concentrations of toxic cells. Fatalities and severe illness of livestock (including birds), pets, wildlife and fish from heavy growths of waterblooms of cyanobacteria are known to occur in almost all countries of the world. Most poisonings occur among terrestrial animals that drink infested freshwater supplies, but marine animals, especially maricultured fish, are also affected (Carmichael and Falconer, 1993; Lambert *et al.*, 1994; Anderson *et al.*, 1993). Predominantly the toxins of freshwater cyanobacteria are of greatest concern to public health and the more common freshwater species responsible for toxic episodes are described in Chapter 19. Nonetheless, in the marine environment toxic cyanobacteria have been found to be responsible for dermatitis and gastrointestinal illness in humans (Moore *et al.*, 1993) while the presence of cyanobacterial toxins in marine fish (Endean *et al.*, 1993), shellfish (Chen *et al.*, 1993) and edible seaweed (Nagai *et al.*, 1996) indicate the potential involvement in food poisoning. As more is known about toxic events associated with marine blooms, further investigations have revealed a growing list of marine cyanotoxins and bioactive compounds (Namikoshi and Rinehart, 1996; Orjala and Gerwick, 1997; Burja *et al.*, 2001). The importance of these organisms in their contribution to toxic marine events is apparent in their impact on both free-living and cultured marine animals, marine tourism and human health. Conversely, the growing pool of toxins from marine cyanophytes offers a unique source of bioactive metabolites for use in medical research (Fujiki *et al.*, 1993; Burja *et al.*, 2001). For these reasons, interest is growing in the culture of marine cyanobacteria and the characterization of their toxins. This chapter focuses on the toxins produced by marine and brackish water species of cyanobacteria.

11.2 Classes of marine cyanotoxins

Although there are over 50 species of cyanobacteria responsible for toxic episodes in both marine waters and freshwaters (Chapter 19), toxic strains within species of *Lyngbya*, *Nodularia*, *Aphanizomenon*, *Trichodesmium*, *Schizothrix* and *Umezakia* are primarily responsible for most marine cyanotoxic episodes. Blooms of *Lyngbya*

majuscula and *Trichodesmium* spp. have been found to be neurotoxic (Nogle *et al.*, 2001; Endean *et al.*, 1993) while hepatotoxins are produced by *Umezakia natans* (i.e. cylindrospermopsin) and *Nodularia spumigena* (i.e. nodularins) (Table 11.1). A number of dermatotoxic compounds are produced by selected genera of marine cyanobacteria (Table 11.1) while many species of cyanobacteria produce pyrogenic endotoxic lipopolysaccharides (Weckesser and Drews, 1979).

11.2.1 Contact irritants

Marine blooms of cyanobacteria are often responsible for contact irritation and gastrointestinal illness in humans. For example, reports of contact dermatitis in ocean swimmers (otherwise known as 'swimmer's itch' or seaweed dermatitis) attribute it to *L. majuscula* (Sims and Zandee van Rilland, 1981). The brominated alkaloid aplysiatoxin and its derivatives (Fig. 11.1) produced by this species cause an inflammatory response on contact with the skin. Aplysiatoxins have also been found to be responsible for severe food poisoning episodes in humans after ingestion of the red alga *Gracilaria* spp. and it is proposed that this may be attributable to the presence of epiphytic *L. majuscula* (Nagai *et al.*, 1996). Ito and Nagai (2000) reported that

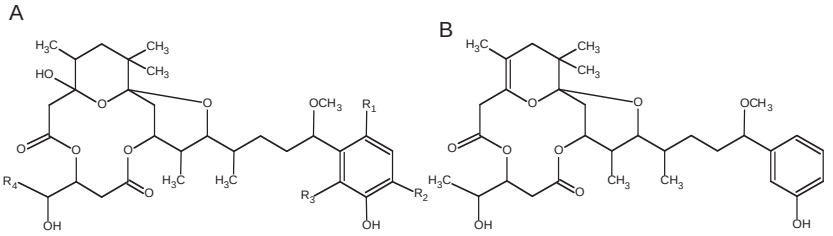
TABLE 11.1 Cyanotoxins from the marine environment

Type of toxin	Source	Reference
Contact irritants		
aplysiatoxins	*Lyngbya, Schizothrix, Oscillatoria*	Nagai *et al.* (1996)
lipopolysaccharides	Most species	Weckesser and Drews (1979); Keleti and Sykora (1982)
lyngbyatoxins	*Lyngbya*	Fujiki *et al.* (1993)
oscillatoxins	*Schizothrix, Oscillatoria*	Mynderse and Moore (1978)
Neurotoxins		
antillatoxins	*Lyngbya*	Orjala *et al.* (1995*b*); Li *et al.* (2001); Nogle *et al.* (2001)
kalkitoxin	*Lyngbya*	Wu *et al.* (2000)
N-sulfocarbamoyl saxitoxins and gonyautoxin-3	*Trichodesmium*	Hawser *et al.* (1991); Jackson *et al.* (2001)
Hepatotoxins		
cylindrospermopsin	*Umezakia*	Harada *et al.* (1994)
microcystins	*Trichodesmium*? and other sources	Shaw *et al.* (2001); Chen *et al.* (1993)
motuporin (nodularin-V)	Cyanobacterial symbiont with *Theonella swinhoei*?	Silva *et al.* (1992)
nodularins	*Nodularia*	Sivonen *et al.* (1989)

food poisoning cases involving *Gracilaria* spp. caused severe illness in almost all patients and resulted in several deaths. Patients reported symptoms including abdominal pain, paraesthesia and burning sensations in the throat and mouth, diarrhea, vomiting, loss of consciousness, convulsions and low blood pressure (Ito and Nagai, 2000). In mice, lethal doses of aplysiatoxins cause bleeding from the small intestine and death results from haemorrhagic shock (Ito and Nagai, 1998). The aplysiatoxins are also potent tumour promoters, as are the structurally related irritant oscillatoxins (Fujiki and Sugimura, 1987) (Fig. 11.1), which have been isolated from blooms of *Oscillatoria nigroviridis* and *Schizothrix calcicola* (Mynderse and Moore, 1978).

Lyngbyatoxin A, produced by *L. majuscula*, is a dermatotoxic alkaloid with identical structure to teliocidin A-1 (Fig. 11.2), a potent skin irritant and tumour promoter from the actinomycete *Streptomyces mediocidicus* (Fujiki *et al.*, 1993). This compound shows similar toxicity in mouse bioassays to the aplysiatoxins (LDmin approximately 0.3 mg kg-1: Gorham and Carmichael, 1979). Lyngbyatoxins B and C (Fig. 11.2), also from *L. majuscula*, are assumed to be weaker tumour promoters than lyngbyatoxin A due to their weaker binding to phorbol ester receptors (Fujiki *et al.*, 1993).

Lipopolysaccharides (LPSs) form part of the cell wall structure of Gram negative bacteria, including cyanobacteria, and can cause allergenic, inflammatory and pyrogenic responses in humans and other animals on contact (Weckesser and Drews, 1979). It is primarily the fatty acid moiety of LPSs that elicits this effect and, as LPS structure may vary phylogenetically, some species tend to be more toxic than others (Weckesser and Drews, 1979). The cyanobacterial endotoxin LPSs are generally less toxic than those of pathogenic bacteria but are a common cause of irritation occurring as a result of contact with ocean blooms of cyanobacteria.



A.

	R_1	R_2	R_3	R_4
aplysiatoxin	Br	H	H	CH_3
bromoaplysiatoxin	Br	Br	H	CH_3
dibromoaplysiatoxin	Br	Br	Br	CH_3
debromoaplysiatoxin	H	H	H	CH_3
oscillatoxin A	H	H	H	H

B. anhydrodebromoaplysiatoxin.

Figure 11.1
The general structure of the contact irritant aplysiatoxins and oscillatoxins-A.

11.2.2 Neurotoxins

Marine cyanobacterial neurotoxins, although apparently common, tend not to be the predominant cause of illness associated with bloom contact. However, the neurotoxins produced by these organisms could accumulate in edible fish (Endean *et al.*, 1993), therefore making them a potential hazard to humans.

Several neurotoxic compounds from marine cyanobacteria have so far been described from *L. majuscula* (Table 11.1) and more recently in *Trichodesmium* spp. (Jackson *et al.*, 2001). The neurotoxic antillatoxins (Fig. 11.3) are cyclic lipotripeptides and potent activators of voltage-dependent sodium channels (Li *et al.*, 2001; Nogle *et al.*, 2001). Antillatoxin has been shown to be neurotoxic in rat cerebellar granule cells (Berman *et al.*, 1999) and both antillatoxin and antillatoxin B are potent ichthyotoxins (Orjala *et al.*, 1995*b*; Nogle *et al.*, 2001). *In vitro*, antillatoxin causes rapid neurotoxicity and neuronal necrosis in rat cerebellar neurons via an *N*-methyl-D-aspartate receptor mechanism (Berman *et al.*, 1999).

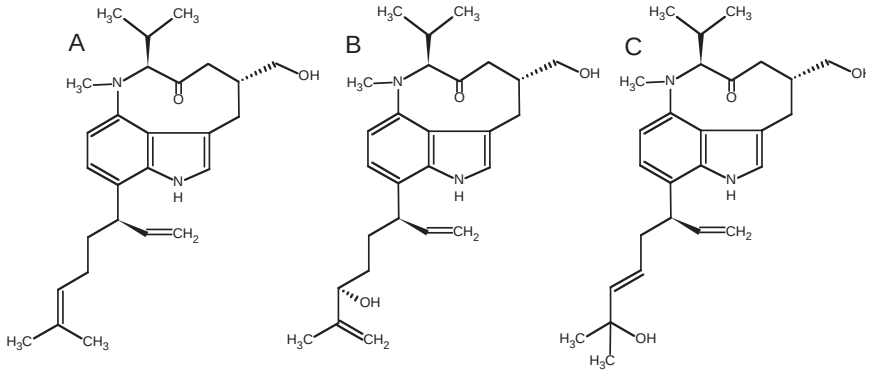
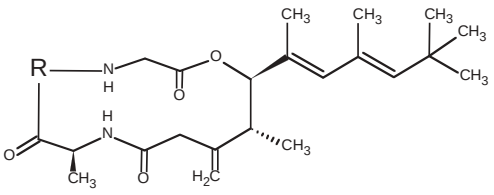


Figure 11.2
The structure of the dermatotoxic alkaloid lyngbyatoxins A, B and C. Lyngbyatoxin A is identical in structure to teliocidin A-1 from *Streptomyces mediocidicus*.



	R
antillatoxin	*N*-methylvaline
antillatoxin B	*N*-methylhomophenylalanine

Figure 11.3
General structure of antillatoxins.

Kalkitoxin (Fig. 11.4), also from *L. majuscula*, is a thiazoline-containing lipid neurotoxin with ichthyotoxic affects and shows delayed neurotoxicity toward rat cerebellar granule neurons. Like antillatoxin, kalkitoxin neurotoxicity is mediated by an *N*-methyl-D-aspartate receptor mechanism but the delayed toxicity suggests that this may be due to a secondary endogenous excitotoxic mediator (Berman *et al.*, 1999).

Neurotoxic factors from *Trichodesmium* spp. have recently been elucidated as *N*-sulfocarbamoyl toxins and gonyautoxin-3 (Jackson *et al.*, 2001); both paralytic shellfish poisons (PSPs) more commonly associated, in the marine environment, with toxic dinoflagellates (see Chapter 7). These compounds (Fig. 11.5) are related to the saxitoxin group of neurotoxins, which are also found in several species of freshwater cyanobacteria (Sivonen and Jones, 1999). Saxitoxins are a group of carbamate alkaloids with potent sodium-channel blockage capabilities and have been implicated in human deaths (Anderson, 1994).

11.2.3 Hepatotoxins

The earliest scientific report of cyanobacterial toxicosis resulting in stock deaths was attributed to the brackish water cyanobacterium *Nodularia spumigena*, after animals drank from Lake Alexandrina in South Australia (Francis, 1878). The toxic factors produced by this species have since been identified as nodularins (Fig. 11.6), cyclic pentapeptide hepatotoxins (Sivonen *et al.*, 1989). As *Nodularia* spp. occur in brackish waters and often in enclosed bays and estuaries, the poisoning of terrestrial

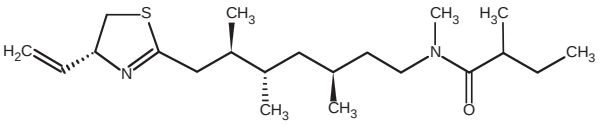
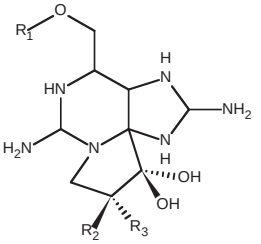


Figure 11.4
Kalkitoxin. A thiazoline-containing lipid neurotoxin from *Lyngbya majuscula*.

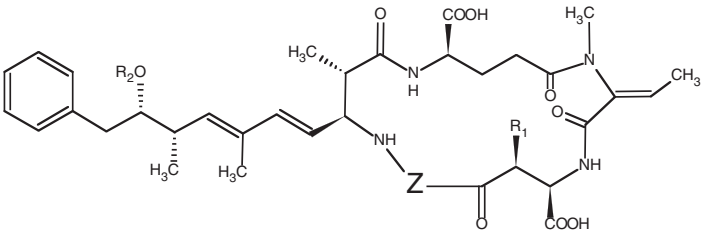


	R_1	R_2	R_3
Sulfocarbamoyl saxitoxins	$CONHSO_3$	OSO_3^- or H	OSO_3^-
Gonyautoxin-3	$CONH_2$	OSO_3^-	H

Figure 11.5
General structure for saxitoxins so far described from marine cyanobacteria.

animals often occurs as a result of drinking from wind-blown scum of this organism that has accumulated on the shoreline. The nodularins are structurally similar to the cyclic heptapeptide microcystins, which are found predominantly in freshwater species of cyanobacteria (Sivonen and Jones, 1999), although the common variant microcystin-LR (Fig. 11.7) has also been found in the marine environment (Chen *et al.*, 1993). More recently, compounds with microcystin-like properties have been investigated in *Trichodesmium* spp. (Shaw *et al.*, 2001). The toxic nodularin analogue motuporin (nodularin-V; Fig. 11.6) has been isolated from the marine sponge *Theonella swinhoei* and there is speculation that this compound is produced by cyanobacterial symbionts within the sponge (Silva *et al.*, 1992).

Both nodularins and microcystins cause liver damage in animals (including humans), primarily through potent and specific inhibition of protein serine/threonine phosphatases (PP) 1 and 2A (MacKintosh *et al.*, 1990; Runnegar *et al.*, 1993). In hepatocytes, PP inhibition leads to hyperphosphorylation of intermediate cytoskeletal



	R_1	R_2	Z
motuporin	CH_3	CH_3	L-valine
nodularin	CH_3	CH_3	L-agrinine
D-Asp[1]nodularin	H	CH_3	L-agrinine
DMAdda[3]nodularin	CH_3	H	L-agrinine

Figure 11.6
General structure of motuporin and nodularins.

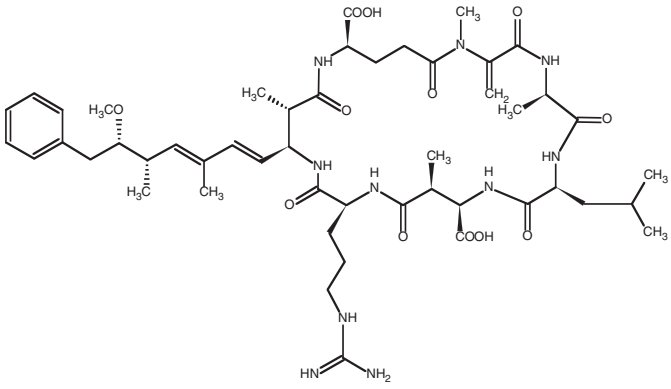


Figure 11.7
The structure of microcystin-LR.

filaments and subsequent cell disruption (Falconer and Yeung, 1992). Orally ingested microcystins and nodularins are rapidly taken up and localized in the liver (Runnegar *et al.*, 1986) through a multi-specific bile acid transport system present in the small intestine and in hepatocytes (Runnegar *et al.*, 1991; Kuiper-Goodman *et al.*, 1999). Once cell disruption occurs, hepatocyte disaggregation leads to hepatic haemorrhage and rapid death (Carmichael, 1994). There is also some evidence of carcinogenic and tumour-promoting effects of these hepatotoxins in mice (Kuiper-Goodman *et al.*, 1999). Due to the potent toxicity of microcystins and their presence in potable waters, a provisional tolerable daily intake (TDI) for microcystins has been set by the World Health Organization at 0.04 µg microcystin kg-1 body weight day-1 (Falconer *et al.*, 1999; Kuiper-Goodman *et al.*, 1999). This corresponds to a TDI of 1.0 µg microcystin l-1 in water containing cyanobacterial cells (Falconer *et al.*, 1999).

Cylindrospermopsin is a cyclic guanidine alkaloid hepatotoxin (Fig. 11.8) found in the brackish water cyanobacterium *Umezakia natans* (Harada *et al.*, 1994) and several species of freshwater cyanobacteria (Sivonen and Jones, 1999). Cylindrospermopsin specifically inhibits glutathione synthesis and generally inhibits protein synthesis (Runnegar *et al.*, 1994, 1995; Terao *et al.*, 1994). This leads to hepatocyte necrosis and lipid accumulation in the liver as well as necrotic effects on other organs, especially the kidneys (Terao *et al.*, 1994). Cylindrospermopsin has been found to accumulate in the flesh of aquacultured Redclaw crayfish (Saker and Eaglesham, 1999) and several cases of human and domestic animal poisonings have been reported (Kuiper-Goodman *et al.*, 1999; Murphy and Thomas, 2001).

11.2.4 Other toxins

Several other toxins from marine cyanobacteria have been identified, although their role in toxic marine events is less well documented. Gerwick *et al.* (1992) describe the cytotoxic undecapeptide hormothamnin A from the marine cyanobacterium *Hormothamnion enteromorphoides*. It has been reported that juvenile rabbitfish feed on *H. enteromorphoides* and that this and other cyanotoxins may act as feeding deterrents (Nagle and Paul, 1998). Hormothamnin A is closely related to the laxaphycins from *L. majuscula* (Bonnard *et al.*, 1997). The barbamides (molluscicidal lipopeptides: Orjala and Gerwick, 1996), malyngamides (ichthyotoxic lipopeptides: Wu *et al.*, 1997), and curacin-A (an antimitotic colchicine analogue: Gerwick *et al.*, 1994; Nagle *et al.*, 1995) are more prominent compounds from a growing number of toxins that have been isolated from *L. majuscula* (see Burja *et al.*, 2001, for a comprehensive review of bioactive compounds from marine cyanobacteria).

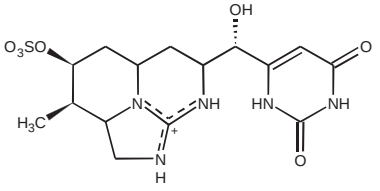


Figure 11.8
The structure of the hepatotoxin cylindrospermopsin.

11.3 Analysis of marine cyanotoxins

In recent years a cooperative effort among cyanotoxin researchers has seen the development of more standard procedures for the analysis of toxic compounds of cyanobacterial origin. Although most routine analysis is carried out for freshwater cyanotoxins, the lessons learnt from the freshwater field can be successfully applied to marine cyanotoxins. Harada *et al.* (1999) provide a detailed list of procedures for the extraction and analysis of the more common cyanotoxins (especially microcystins and nodularins) and a comparison of the various analysis methods currently available. A recent analysis of methods for the extraction and analysis of nodularins and a proposed pathway for analysing both freshwater and marine cyanotoxins is described by Dahlmann *et al.* (2001) (Fig. 11.9).

11.3.1 Sample collection and storage

Collection of waterbloom samples is with plastic or glass containers. For toxicity studies using a mouse bioassay about 0.5 g dry weight of cells is required for a statistically significant LD50. This usually means collecting 1–2 l of light to moderate waterbloom to as little as 50–100 ml of a heavy surface accumulation. For the newer, more sensitive assays only a few millilitres of bloom sample are needed.

It is important that where possible an attempt should first be made to identify the species of cyanobacteria involved by microscopic analysis and taxonomic keys where available (see Chapter 19). This information may indicate the toxins likely to be present and allow investigators to prepare samples appropriately for further analysis. In the case of poisoning events, details of a patient's symptoms might also provide an indication of the toxins present.

For the collection and storage of potentially toxic freshwater cyanobacteria, Harada *et al.* (1999) recommend that collected bloom material preferably be kept refrigerated in the dark prior to analysis within 24 hours in order to prevent toxin breakdown. In the event that samples must be kept for extended periods, it is recommended that they be frozen although this may lead to cell lysis and toxin release.

Where samples contain little cyanobacterial biomass they should be concentrated, particularly if the method to be used for detecting the toxin has low sensitivity. Advantage can be taken of gas-vacuolate cyanobacteria as they will accumulate on the surface of the liquid sample. Concentration of cells, in this case, can be achieved by allowing the sample to settle in a separating funnel and running off the excess liquid. Less buoyant cells can on the other hand be collected by centrifugation or by allowing the sample to settle. If a relationship between cyanobacterial biomass and toxin amount is required, the preferred method of concentration is by filtration using the method outlined in detail by Harada *et al.* (1999). Thus, where the ingestion of brackish water cyanobacteria (e.g. *Nodularia* or *Umezakia*) is concerned this may be the most appropriate method for concentration of cyanobacterial samples. Alternatively, frozen samples can be freeze-dried and stored in a cool, dry place until required for analysis. Material prepared in this way can also be weighed to determine cyanobacterial biomass prior to extraction. Whatever method of cell concentration is chosen samples should either be processed as soon as possible or kept in a dried form and stored in a cool dry place (e.g. a vacuum desiccator) for later analysis.

If the samples are seafoods or other sources potentially containing marine cyanotoxins (e.g. sea sponges) then haste is also required to prevent breakdown of the toxin

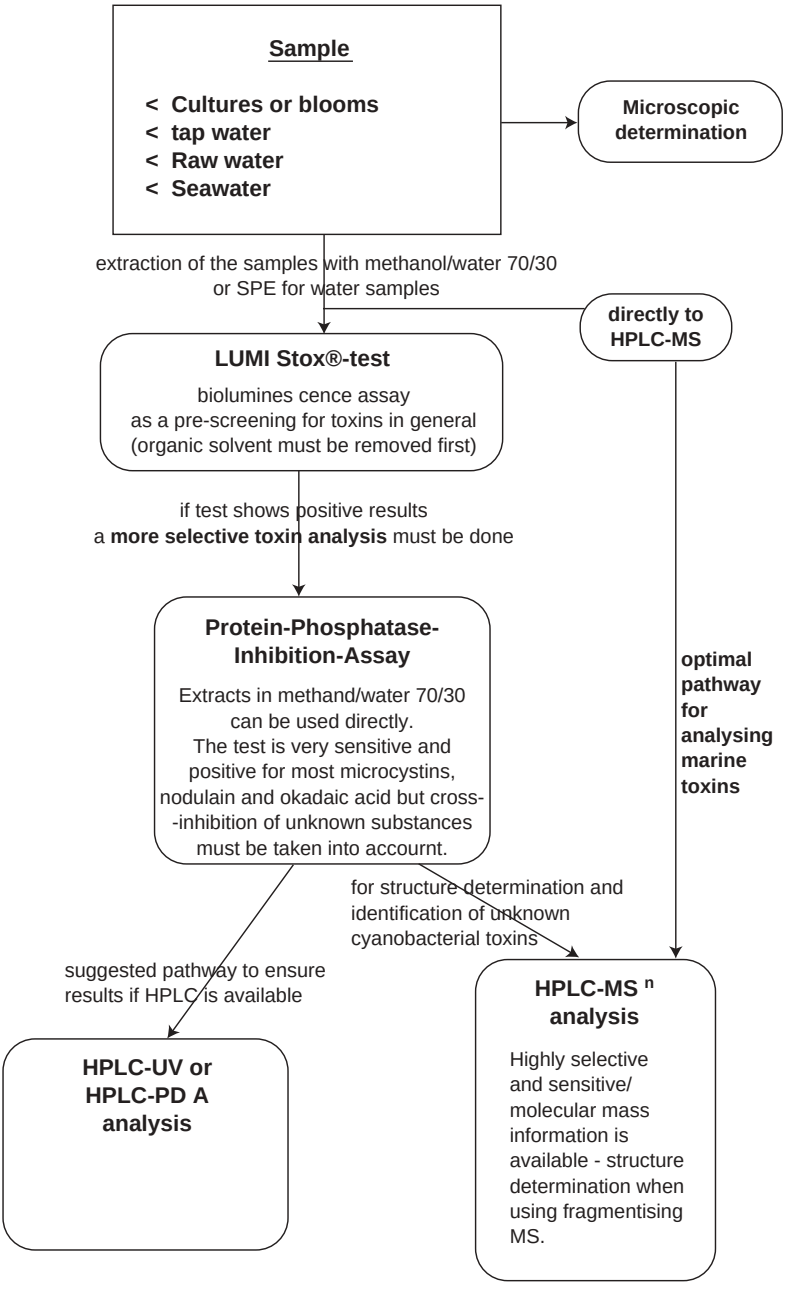


Figure 11.9
Suggested pathway for analysing cyanobacterial toxins. Reprinted from Toxicon, Vol. 39, Dahlmann et al., Different methods for toxin analysis in the cyanobacterium *Nodularia spumigena* (Cyanophyceae), 1183-1190, Copyright 2001, with permission from Elsevier Science.

before analysis. Storage must also ensure that metabolism of cyanotoxins does not occur. Freeze-dried powders of such material will keep for extended periods before analysis and have been used successfully in the analysis of cylindrospermopsin in fish and crayfish tissues (Saker and Eaglesham, 1999). Further diagnosis of the toxin involved, or characterization of its histological effects, can be aided by collecting and preserving organs of affected animals during necropsy for later microscopic analysis.

It is important that investigators should evaluate their collection and storage methods with respect to the cyanotoxin of interest.

11.3.2 Cyanotoxin extraction and preparation for analysis

The method used for cyanotoxin extraction will depend largely on the specific toxin or toxins to be investigated and the type of material containing the toxin (e.g. cyanobacterial biomass or animal tissues). For most cyanotoxins, researchers utilize organic solvent extraction and sample clean-up using various chromatographic techniques. However, many compounds vary in their solubility in different solvents (Lawton and Edwards, 2001) and their extraction may also depend on the matrix in which they are held. Thus, extraction methods must be evaluated if they are to be used routinely. Currently there are few generalized methods for the extraction and preparation of marine cyanotoxins, although specific methods can be obtained from the literature. Several authors (e.g. Harada, 1995; Lawton and Edwards, 2001; Dahlmann *et al.*, 2001) have developed generalized procedures for the extraction and analysis of the more common cyanotoxins (including nodularins), which can also be applied to marine cyanotoxins (Fig. 11.9).

Depending on the quality of the toxic extract, further analysis may be hampered by extraneous organic material and sample clean-up may be required. Sample clean-up removes unwanted material from the extract while retaining the compound of interest and can be used to enrich cyanotoxins where concentrations are extremely low. High-performance liquid chromatography (HPLC) analysis of microcystins and nodularins, for example, benefits from post-extraction clean-up of the sample using C-18 silica-based cartridge systems. Harada *et al.* (1999) recommend sample clean-up in cases where low concentrations of cyanotoxins are expected, identification and quantification are required, or for bioassays in which other materials might affect the outcome. A detailed procedure for the concentration and clean-up of microcystins and nodularins from water samples is supplied by Harada *et al.* (1999).

Extraction methods for specific cyanotoxins may vary and methods must be evaluated if they are to be used for the quantification of the toxin to be investigated. The antillatoxins have been successfully extracted from dried and alcohol-preserved cyanobacterial material using dichloromethane: methanol (2: 1) and subsequent fractionation using silica-gel chromatography and HPLC (Orjala *et al.*, 1995*b*; Nogle *et al.*, 2001). In order to obtain yields of antillatoxins to allow chemical analysis (milligram quantities) relatively large amounts of cyanobacterial material were required (several hundred grams) in both cases. Similar methods have been applied to the purification of hormothamnin A from *Hormothamnion enteromorphoides* (Gerwick *et al.*, 1992), kalkitoxin from *Lyngbya* (Wu *et al.*, 2000), aplysiatoxins from the seaweed *Gracilaria* (Nagai *et al.*, 1996) and oscillatoxins from an *Oscillatoria nigroviridis*/*Schizothrix calcicola* bloom (Mynderse and Moore, 1978). The use of organic solvents in the extraction of cyanotoxins is favoured as they are often easily evaporated, allowing concentration of the sample. Alternatively, cylindrospermopsins have

been extracted from freeze-dried fish and crayfish tissues in water by sonication (Saker and Eaglesham, 1999) while some cyanotoxins have been extracted from frozen bloom material by several cycles of freeze-thawing followed by extraction into an appropriate solvent. Keleti *et al.* (1979) extracted LPS from several species of cyanobacteria using hot phenol/water extraction followed by enzymatic hydrolysis of glucan. Whichever method of extraction is used, it is important to determine solvent effects in subsequent bioassays or physico-chemical analysis.

11.3.3 Cyanotoxin analysis using bioassays and biochemical methods

A wide variety of bioassays is now available for the analysis of natural toxins (see Harada *et al.*, 1999, for a review). Commonly, marine cyanotoxins are examined for their toxicity using the brine shrimp (*Artemia salina*) assay, which offers a relatively cheap and simple test for toxicity. The assay can use crude or purified extracts of cyanotoxins to determine the concentration that results in mortality of 50% of brine shrimp larvae (LD50). Results can be obtained within 24 hours. This assay is also commonly used for the determination of many freshwater cyanotoxins and is a popular alternative to other bioassays. Kits for brine shrimp assays are now available, although little cost is required to develop the assay with readily available laboratory materials. Harada *et al.* (1999) provide a detailed procedure for the brine shrimp assay as it can be applied to cyanobacterial samples.

For the determination of ichthyotoxicity, Gerwick and co-workers often make use of the goldfish (*Carassius auratus*) toxicity assay of Bakus and Green (1974). This assay examines the behaviour of fish placed into water containing extract or purified toxin. Bakus and Green (1974) defined extracts as highly, moderately or mildly toxic depending on the time taken for fish to die in the presence of the toxin. Lethal dose values (e.g. LD50) can be determined by serial dilution of the extract or purified toxin and observation of the fish for a specified period (e.g. 1 hour). Specific use of this assay for marine cyanotoxins is described by Orjala *et al.* (1995*a*). A similar method is applied for the determination of molluscicidal activity in marine cyanobacterial extracts using the snail (*Biomphalaria glabrata*) toxicity assay of Hostettmann *et al.* (1982).

More commonly used in the analysis of freshwater cyanotoxins, the mouse bioassay provides an indication of toxicity to mammals. Falconer (1993) provides specific details of the mouse bioassay as it is applied to cyanotoxins and further details of the assay can be found in Chapter 13. The mouse bioassay can be used to screen for both neurotoxins and hepatotoxins of cyanobacteria because the signs of poisoning for these two toxin groups are very different. The method is used to determine the minimum amount of toxin required to kill a mouse (the LD50 or LDmin) or it can be used to measure PSPs of cyanobacteria by the standard AOAC (1990) method. The major disadvantages of the test are that it does not detect toxins at low concentrations (Harada, 1995) especially in finished drinking waters, and has limited application at water utilities which often have no facilities for maintaining experimental animals. The mouse bioassay does however remain a useful screening method for the neurotoxic freshwater cyanotoxins anatoxin-a and -a(s), which have no other rapid screen available. This assay is more costly than many other bioassays, often requires a licence, and is not allowed in some countries. Nonetheless, the mouse bioassay has been used for the analysis of several marine cyanotoxins, including aplysiatoxins (Nagai *et al.*, 1996).

Mouse bioassays have also been employed for the analysis of cyanobacterial LPS toxicity (Katz *et al.*, 1977). However, the biological activity of LPSs has also been assessed using the Schwartzman test (using rabbits), the *Limulus* amoebocyte lysate test and the ligated rabbit ileal loop assay (Keleti *et al.*, 1979; Keleti and Sykora, 1982).

Several methods are available for the determination of toxicity using isolated mammalian cells. For example, a number of marine cyanotoxin studies have used isolated rat cerebellar granule neurons for neurotoxicity assays (e.g. Berman and Murray, 1996; Berman *et al.*, 1999; Li *et al.*, 2001). The release of lactate dehydrogenase (LDH) from these cells has been used as an indicator of toxicity (Berman *et al.*, 1999). The use of isolated hepatocytes for toxicity testing also provides specific and accurate testing for hepatotoxic cyanotoxins and can be applied to the analysis of microcystins and nodularins (Heinze, 1996). Cyanotoxins with sodium-channel blocking activity can also be assayed using neuroblastoma cells as an alternative to mouse bioassays or using the methods of Jellet *et al.* (1992) and Manger *et al.* (1995).

There are now a number of enzyme-linked immunosorbent assay (ELISA) kits available for the analysis of microcystins. Although specific to this common freshwater cyanotoxin, these ELISA kits can be applied to nodularins and possibly motuporin. Application of this technology to marine cyanotoxins is limited, although several methods are described for some marine toxins in Chapter 12.

Several biochemical assays based on PP inhibition have been developed for the analysis of nodularins and microcystins. While older PP inhibition assays utilize 32P-labelled phosphorylase *a* as a substrate (e.g. Honkanen *et al.*, 1995), several alternative methods using colorimetric (An and Carmichael, 1994; Ash *et al.*, 1995) or fluorimetric (Fontal *et al.*, 1999; Long, 2001) techniques are now available. The PP inhibition assays are both highly sensitive and selective for this group of cyanotoxins and capable of detecting them in picogram quantities (Harada *et al.*, 1999). Cyanotoxins in the saxitoxin group of compounds have been successfully analysed using sodium-channel and saxiphilin competitive binding assays (Carmichael *et al.*, 1997). Li *et al.* (2001) used a batrachotoxin binding assay to analyse the binding of antillatoxin to cerebellar granule-cell sodium channels. Similar methods can be used to analyse a number of neurotoxic cyanotoxins.

11.3.4 Physico-chemical methods for cyanotoxin analysis

Purified toxin in microgram quantities or greater are usually required for complex chemical characterization of cyanotoxins. Generally, structural identification can be made using a combination of mass spectrometry (e.g. fast atom bombardment mass spectrometry – FABMS), nuclear magnetic resonance (NMR), infrared (IR) and ultraviolet (UV) spectrometry on the purified compound. Specific identification and absolute configuration is often achieved through organic synthesis and comparison with the purified compound. Such physical data can be valuable in developing analytic procedures as characteristic MS fragments or specific absorption maxima can be determined. With this in mind, where routine analytical procedures have not been developed, but cyanotoxins have been characterized, reference should be made to the specific literature available.

Unlike freshwater cyanotoxins, few routine physico-chemical analysis methods currently exist for the determination of marine cyanotoxins. However, the processes that have been developed for the analysis of freshwater cyanotoxins can in many

cases be applied directly to marine cyanotoxin analysis. Harada *et al.* (1999) provide detailed descriptions of some physico-chemical methods currently used for the analysis of several cyanotoxins including nodularins, microcystins, saxitoxins and cylindrospermopsin. A suggested pathway for the analysis of both marine and freshwater cyanobacterial toxins is provided in Fig. 11.9.

Following sample clean-up, identification and analysis of cyanotoxins use several types of standard analytical procedure. Primarily, HPLC or gas chromatography (GC) methods are used to separate toxins from within a complex mixture, but detection methods may vary. For saxitoxin and their derivatives in freshwater cyanobacteria, the post-column fluorescence HPLC method of Oshima et al (1993) has been used for analysis of Australian *Anabaena circinalis* (Humpage *et al.*, 1994; Negri and Jones, 1995; Negri *et al.*, 1995) and *L. wollei* (Carmichael *et al.*, 1997). HPLC analysis of aplysiatoxins can be carried out using UV detection (Nagai *et al.*, 1996). The popularity of liquid chromatography-mass spectrometry (LC-MS) systems has meant that many investigators can now easily identify their analytes in complex mixtures without derivatization and with greater accuracy. Saker and Eaglesham (1999), for example, used LC-MS for the analysis of cylindrospermopsin. As microcystins and nodularins are the most common cyanobacterial toxins present in water supplies, they have been the subject of several analysis methods. These methods include gel chromatography, thin-layer chromatography, HPLC, MS and NMR. Additional detectors for HPLC include photodiode array (PDA), fluorescence (FL) and chemiluminescence (CL). The use of PDA detection is particularly useful in identifying compounds with specific UV/vis spectra or known UV/vis absorption maxima. Cylindrospermopsins, nodularins and microcystins have been analysed using this technique (Harada *et al.*, 1999; Hawkins *et al.*, 1997). PDA detection is now commonly used in conjunction with LC-MS to aid in the identification of compounds. FL and CL detectors are often utilized in pre-column derivatization procedures (e.g. for the detection of dansyl-cysteine adducts of microcystins: Harada, 1995). For a more detailed description of the methods for detection and analysis of cyanotoxins, refer to Harada *et al.* (1999).

Physico-chemical analysis of cyanotoxins is of particular use in cases where different toxin groups (or variants of the same toxin group) may be present within an extract. This is of particular importance if the samples are also used for bioassays that could be complicated by the presence of more than one toxin. Samples of *L. majuscula*, for example, may contain a number of unrelated cyanotoxins (Yokokawa *et al.*, 2000; Burja *et al.*, 2001). Physico-chemical analysis also provides the benefit of accurate quantification compared with many bioassays, especially where toxin concentrations are low. Depending on the method used, physico-chemical analysis also allows high throughput of samples, which is of particular interest to investigators carrying out routine analysis.

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12

In vitro assays for phycotoxins

A. D. Cembella, G. J. Doucette and I. Garthwaite

12.1 Introduction

The development of *in vitro* techniques, including immunoassays, enzyme assays, receptor assays and cytotoxicity tests, offers valid approaches to detection of phycotoxins in complex organic matrices, such as extracts of shellfish tissues and toxic microalgae. In principle, such methods have several inherent advantages over sophisticated chemical analytical alternatives (see Chapters 7–10) for routine phycotoxin monitoring. Certain configurations yield extremely high sensitivity (theoretical detection limit typically $<10^{-12}$ M) and specificity towards the target toxin analyte, for a comparatively low investment in hardware, operating cost and technical training.

In vitro diagnostic test kits are expensive to develop; however, when manufactured in quantity, the cost per analysis becomes very competitive with other assays and analytical methods. In many cases, relatively crude extracts may be assayed, without resorting to the extensive clean-up procedures often required for instrumental analysis by physico-chemical methods. By comparison, most advanced instrumental chemical analyses can only be performed in centralized laboratories, due to the complexity of operation and the requirement for sample clean-up. Biochemical and cytological assays may be used by mariculturists and regulatory personnel as a harvest management tool for detecting phycotoxins in shellfish, finfish, and natural assemblages of toxic phytoplankton. Direct detection of phycotoxins in plankton collected from the water column would serve as an early warning system for aquaculture and wild harvest operations. As rapid detection kits, such assays can be conducted by field technicians on board ship, at dockside, at aquaculture sites or in local laboratories. For preliminary phycotoxin screening, mariculturists and fishermen might choose to perform assays on landing their catch, as extraction and preparation of samples for these techniques is often rudimentary. Mass screening with *in vitro* assays over appropriate temporal and spatial scales might also be useful in establishing selection criteria for aquaculture sites.

With microtitre plates and automated plate readers, *in vitro* microassays can accommodate the rapid parallel screening of large sample numbers (>100 per day), whereas most analytical detection methods are limited to sequential sample injection, even when equipped for automated operation. In many regulatory programmes, the phycotoxins of concern are well known and resolution of each individual component in a suite of related toxins may not always be necessary. Thus, a rigorously validated, well-calibrated *in vitro* assay can serve as the first line of defence for phycotoxin monitoring, with only key samples being subjected to further analysis by

instrumental methods for confirmation or structural elucidation. *In vitro* diagnostic methods may be particularly useful for toxin screening in the plankton, especially where both toxic and non-toxic phytoplankton strains of the same species co-occur, or when toxic species cannot easily be identified based on morphological criteria.

Cell culture (cytotoxicity) bioassays, immunoassays, receptor assays, and enzymatic tests are less-controversial alternatives to bioassays using live mammals (see Chapter 13), which are increasingly subject to legislative restriction for routine toxin monitoring because of animal rights concerns. The dependence on mammalian bioassays to yield 'biologically relevant' toxicity data might be significantly reduced without compromising public health and seafood safety by employing *in vitro* assays. Such assays can be performed using simpler extraction procedures as they have greater specificity than whole-animal assays and do not require the removal of agents such as heavy metals, which can contribute to false positives.

In vitro assay methods may be broadly categorized into two general subtypes – functional and structural assays. Functional assays are based on the biochemical action of the toxin (e.g. binding to the ion channels of receptors), and hence quantitation tends to correlate well with the specific toxicity of the analyte. Differences with whole-animal responses will exist due to variation in mechanisms of toxin uptake and conversion in the body. Nevertheless, for matrices that contain several toxic components with a similar mode of biological activity, but which vary in specific potency, such assays should yield an accurate estimate of net toxicity.

In contrast, structural assays are dependent on the conformational interaction of the analyte (toxin) with a molecular recognition factor, such as the paratopic binding sites of antibodies in immunoassays. Thus, cross-reactivity in immunoassays is limited to components with compatible epitopic sites and may not reflect relative biological activity or specific toxicity. Such assays yield only an integrated quantitative value representing a group of toxins, whereas the components may vary widely in specific toxicity. Direct comparison with 'toxicity' as determined from mammalian bioassays may be invalid. The lack of broad-spectrum cross-reactivity for toxic, naturally occurring analogues has been a major drawback to the use of quantitative immunoassays for screening phycotoxins in naturally contaminated samples; however, this is being overcome by second-generation antibodies, where more care is taken in designing the antibody 'receptor site'.

Although the application of *in vitro* assays usually does not require use of sophisticated technology, this is not the case for their development. Costs of trained labour, materials, laboratory facilities, bulk production of diagnostic reagents, mass production of test kits, collaborative testing, patenting, certification, and marketing can often be excessive – perhaps exceeding the potential for cost recovery. There are also inherent limitations of many *in vitro* methods, including the potential to generate false-positive or false-negative responses for target phycotoxins, due to the presence of toxicologically inactive congeners, or failure to detect all toxigenic components in a complex suite of toxin analogues and/or other metabolites. Finally, although availability of certified toxins for preparation and validation of *in vitro* diagnostic techniques has increased in recent years, efforts to develop broad-spectrum assays continue to be limited by the supply of purified phycotoxin analogues.

The importance of determining the degree of specific versus non-specific binding for *in vitro* assay techniques cannot be overemphasized. Non-specific binding (to either antibody or receptor) can be defined as extraneous interaction with the ligand (i.e. toxin) or receptor resulting from the presence of bindable non-target

components in the sample matrix. Spurious binding of components (fatty acids, proteins, etc.) unrelated to the analytes of interest (toxins) can also occur in immunodiagnostic tests. Experimentally, non-specific binding is taken into account by using controls containing a saturating concentration of unlabelled toxin, and the resulting non-specific binding factor is subtracted from results before generating competitive binding curves.

In spite of the attributes of *in vitro* assays, there are surprisingly few of these techniques in routine use for phycotoxin monitoring. The technology is advancing so rapidly, however, that this chapter focuses on techniques that have reached the advanced prototype stage or are in commercial production, rather than providing a review of the latest developments. For detailed comprehensive reviews of emerging and developed technologies, including *in vitro* methods for phycotoxin detection and quantitation, other recent publications (e.g. Towers and Garthwaite, 2001; Van Dolah and Ramsdell, 2001) should be consulted.

12.2 Development and application of immunoassays

In the last few decades, there have been several concerted attempts to produce reliable immunodiagnostic test kits for various phycotoxins. Many of these efforts have been hampered by the lack of purified toxins for conjugation and difficulties in producing stable immunogens from toxins of relatively low molecular weight [e.g. saxitoxin (STX), domoic acid (DA)]. Low molecular weight components with little intrinsic antigenic activity (haptens) must be coupled to a carrier (typically a protein) prior to inoculation. As toxin conjugates for immunization are usually prepared from only a single, readily available derivative, whereas toxigenic phytoplankton and affected target species often contain a suite of chemically related derivatives, cross-reactivity is important in the development of immunological methods. In any case, the sensitivity of immunodiagnostic tests is typically orders of magnitude greater than the corresponding mouse bioassay or high-performance liquid chromatography (HPLC) method – even picogram quantities of toxins are detectable. New and more sophisticated antibody methods that address specificity problems by employing better initial chemistry for hapten production are also becoming available.

Immunoassays for phycotoxin detection have been prepared using both monoclonal and polyclonal antibodies. In general, polyclonal antibodies are more rapidly and inexpensively produced than monoclonals and their affinity for multiple epitopic sites tends to yield a heterogeneous, broad-spectrum assay, translating into superior cross-reactivity among related antigens. Monoclonal antibodies are more suited for single epitope detection and as they are generated from 'immortal' cell lines, they can be consistently produced with low batch-to-batch variability. These antibodies can also be selected to recognize a chemical backbone structure common to all toxin analogues of a group, and may be used in combination to detect a range of congeners in a given toxin class.

A wide array of different assay configurations may be used for immunodiagnostic tests. These include direct and indirect coupling, competitive interaction and 'sandwich' assays, although the low molecular weight of many of the phycotoxins limits the use of 'sandwich' assays, because of steric hindrance of simultaneous

binding of such toxins by two antibodies (see Fig. 12.1). Detection systems for immunoassays commonly make use of a radio-label (RIA), a coupled enzyme reaction (EIA), or a fluorescent marker (FIA), but other detection modes (e.g. chemiluminescence) may also be employed. A protocol for screening shellfish tissues by enzyme-linked immunosorbent assay (ELISA) has recently been advanced by

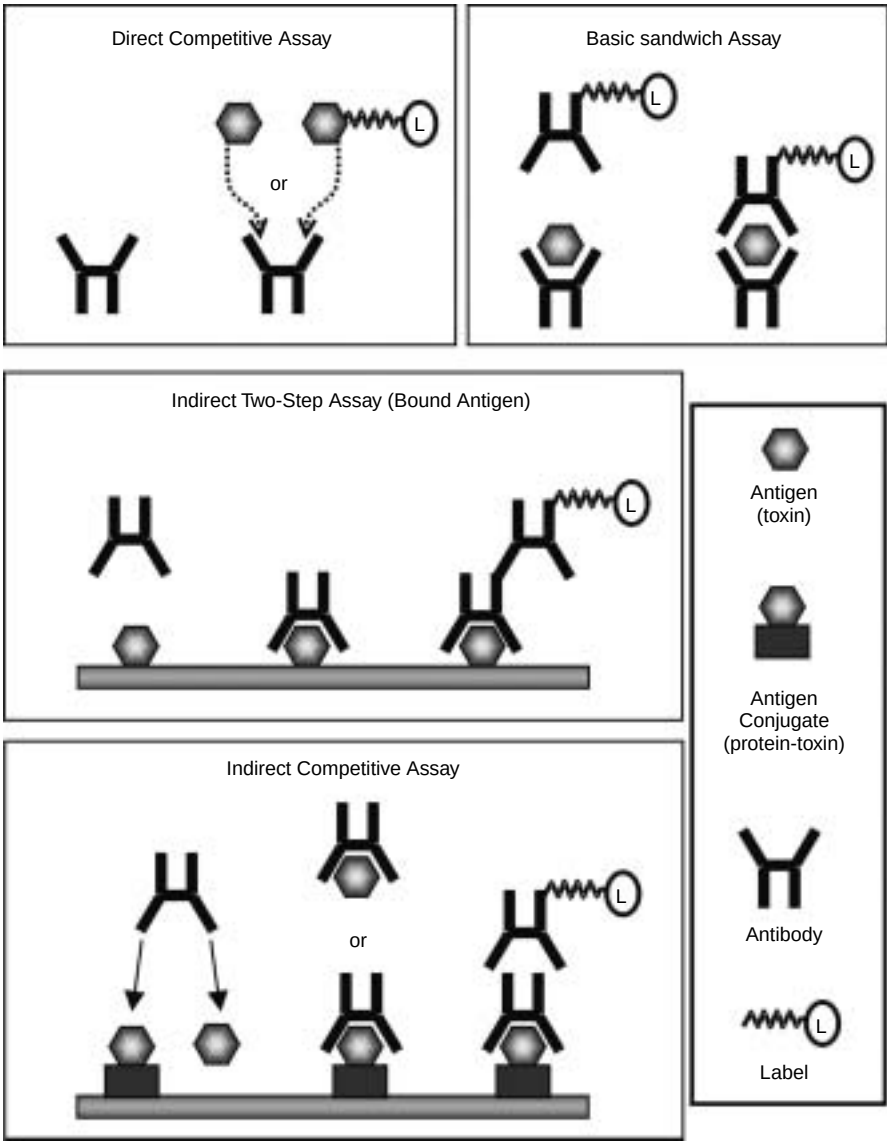

Figure 12.1
Schematic diagram showing alternative immunoassay configurations.

AgResearch, New Zealand, and uses a battery of antibodies with sufficient sensitivity and selectivity to detect all known phycotoxin classes at levels below the regulatory maximum permitted limits (MPLs) (Garthwaite *et al*., 2001).

12.2.1 Immunoassays for PSP toxins (saxitoxin analogues)

Early efforts to produce polyclonal antisera to STX (Johnson *et al*., 1964) using bovine serum albumin (BSA) as the carrier protein yielded a relatively labile conjugate and poor activity. Several RIA methods (Carlson *et al*., 1984; Davio *et al*., 1985) and ELISA assays (Chu and Fan, 1985; Davio *et al*., 1985) described in the literature have employed various cross-linking agents and STX derivatization reactions to produce antibodies. In most cases, toxin conjugates for antibody production were prepared only from STX (or via a synthetic derivative, e.g. saxitoxinol). Where cross-reactivity with other toxins associated with paralytic shellfish poisoning (PSP) has been evaluated, a critical deficiency in most immunoassays has been a weak recognition of the neosaxitoxin (NEO) group of N–1-OH toxins (Carlson *et al*., 1984; Chu and Fan, 1985; Usleber *et al*., 1991). This shortcoming has been addressed in two ways. By combining immunoassay results for STX and NEO, a good correlation with the mouse bioassay may be achieved (Chu *et al*., 1996). Enhanced detection of analogues is also possible by combining multiple antibodies within one ELISA. For a review of immunoassay methods for PSP toxins, see Usleber *et al*. (2001).

An absorption-inhibition ELISA (SAXITOXIN TEST®, Institut Armand-Frappier, Laval, Canada) was the first practical attempt to configure a rapid diagnostic immunoassay kit for PSP toxins. In this assay, STX is immobilized on polystyrene batons to competitively bind free STX-antibody from a toxic sample-antibody incubation mixture, and thus the colourimetric signal intensity of the assay varies inversely with STX concentration. The polyclonal antibody by STX used in the SAXITOXIN TEST® kit exhibits relatively broad antigen specificity and cross-reacts well with at least two gonyautoxins (GTX2 and GTX3), but there is weak detection of the low-potency N-sulfocarbamoyl toxins (Cembella *et al*., 1990). When tested on toxic shellfish against the HPLC method with fluorescence detection and AOAC mouse bioassay, the SAXITOXIN TEST® kit yielded comparable results in the critical toxicity range 400–2,000 µg STXeq kg^{-1} shellfish tissue with no false-positive responses (Cembella and Lamoureux, unpublished data). The polyclonal antibody was also shown to be useful for assaying PSP toxins in phytoplankton cultures and in natural phytoplankton assemblages (Cembella and Lamoureux, 1993). Unfortunately, detailed collaborative studies were not completed and the kit is no longer commercially produced.

A direct EIA prepared from a polyclonal anti-STX antibody, configured as a microtitre plate ELISA and as a test strip assay, by conjugation of STX to horseradish peroxidase, showed high sensitivity (3–4 µg kg^{-1} tissue) for the detection of STX in shellfish (Usleber *et al*., 1991). Comparisons of the effect of heterologous PSP toxin-enzyme conjugates and cross-reactivity with purified toxins (Usleber *et al*., 1994) indicated that the sensitivity of the direct EIA method was generally superior to the indirect technique. A modified assay for qualitative screening employs a membrane filter in a competitive enzyme-linked immunofiltration assay (ELIFA), a simple, rapid assay that can be performed outside of a well-equipped laboratory (Usleber *et al*., 1995). The cross-reactivity to NEO is poor, but STX, GTX2/GTX3, and decarbamoyl (dc-) STX are detectible below 800 µg kg^{-1} shellfish tissue.

A related ELISA method for the detection of STX in shellfish is commercially available as a test kit (RIDASCREEN®, R-Biopharm GmBH, Darmstadt, Germany). This kit has been subjected to collaborative testing against other methods by the Measurement and Testing Programme (BCR) of the European Commission (Van Egmond *et al*., 1994). Not surprisingly, the ELISA test yielded an overestimate of 'STX', when used to assay for STX spiked into a mussel tissue matrix containing several other PSP toxins. This highlights the pitfalls of method comparisons that relate the integrated toxin content for a toxin family to a single toxin measured analytically.

The recently developed MIST Alert™ (Jellett Biotek, Dartmouth, Canada) has been shown to be highly effective for the detection of PSP toxins in shellfish (Laycock *et al*., 2001) and plankton matrices (Silva *et al*., 2001, Cambella *et al*., 2001*a*) (Fig. 12.2). This lateral flow immuno-chromatographic (LFI) assay is available on a platform similar to that of a common home pregnancy test kit and permits screening for PSP toxins in <20 min. The polyclonal antibodies have been well characterized for their cross-reactivity and limit of detection (Fig. 12.3) for multiple PSP toxins standards (CRMP, IMB, National Research Council, Halifax, Canada), and all STX analogues commonly found in shellfish, including the N-sulfocarbamoyl derivatives, are detected, albeit at somewhat reduced sensitivity for the N–1-OH toxins (Laycock *et al*., 2001).

The MIST Alert™ for PSP toxins has been validated with over 2,000 shellfish samples in government regulatory laboratories located in Alaska, Maine and the United Kingdom, as well as at field aquaculture sites (Fig. 12.4). No species-specific artifacts interfered with the test and consistent responses were obtained with all shellfish species tested. These trials have shown the MIST Alert™ to be extremely effective in detecting PSP toxins in shellfish samples, when compared in parallel to the AOAC (2000) mouse bioassay – toxin levels >800 µg STXeq kg^{-1}, as determined by mouse bioassay, always registered as 'positive' according to the immunodiagnostic test. The detection limit for the test is *c.* 400 µg STXeq kg^{-1}, but varies slightly according to the profile of PSP toxin analogues. According to the assay, about 5% of the samples <400 µg STXeq kg^{-1} were 'positive' (below the detection limit of the mouse bioassay and hence false positive) and about 5% of the samples containing between 400 µg STXeq kg^{-1} and 790 µg STXeq kg^{-1} were 'negative'. The test can detect PSP toxins as low as 20 µg STXeq kg^{-1} of tissue. Various toxicity profiles in shellfish samples may change the actual detection limit, as the antibodies in the kit detect individual toxin analogues with different sensitivities.

12.2.1.1 *MIST Alert™ for PSP toxin assay*

12.2.1.1.1 *Shellfish tissues*

Complete details on the application of this assay in the laboratory and directly with field samples are provided in the instruction sheet accompanying the MIST Alert™ kits. Fig. 12.5 is a flow chart for the application of the MIST Alert™ for PSP toxin to field samples of shellfish. The following are basic instructions for performing the rapid tests on extracts prepared according to the standard or modified AOAC (2000) protocol for shellfish tissues (see Appendix 12.1):

1. Take 100 µl of the AOAC tissue extract into the small pipette provided (up to the black line marked on the pipette). Add this to the small vial of running buffer and mix the contents by pipetting up and down in the vial at least three times.

2. Remove the test cassette from the pouch and check that the desiccant pack in the pouch has a blue colour. If the colour has changed to pink, do not use the test. Also, check the expiration date on the foil pouch. The test kits have a shelf life in excess of one year and may be stored at room temperature.
3. Transfer 100 µl of the mixed AOAC extract/buffer solution to the sample (S) well on the test cassette. Mark the cassette with a sample number if running multiple samples.

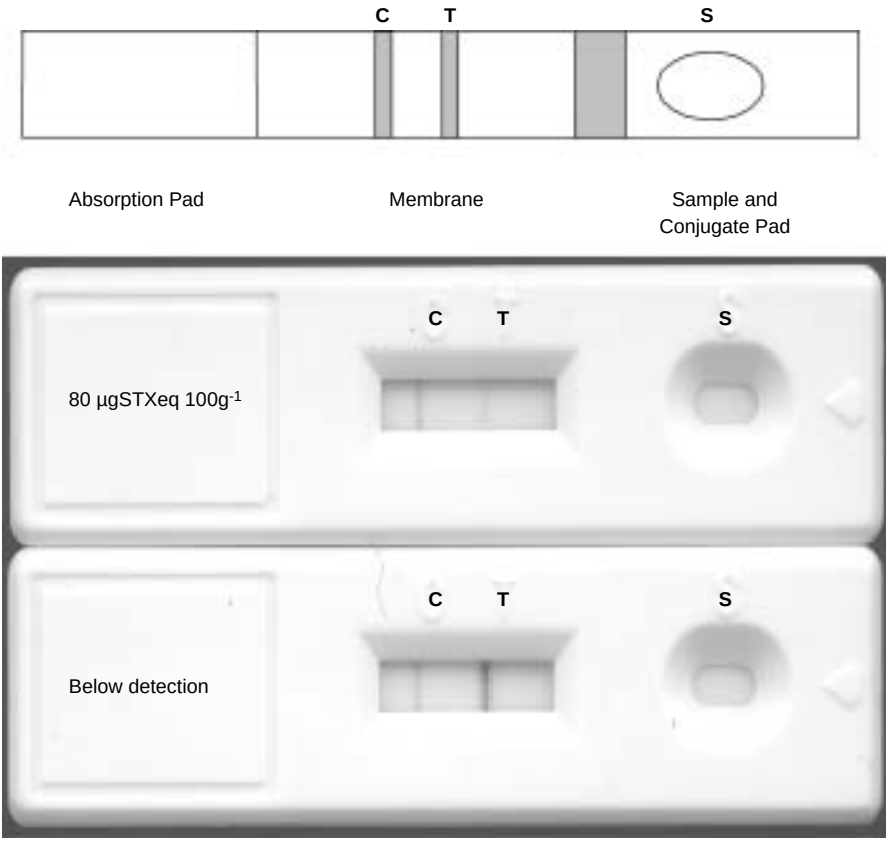

Figure 12.2
Diagram of the MIST Alert™ test strip. The components are aligned with two cassettes containing developed test strips. Strips consist of an absorption pad, a membrane striped with a mixture of toxin analogues (T line on the right) and an antibody-detection reagent (C line on the left), a sample pad, and a conjugate pad containing the antibodies. A visible T line indicates absence of toxin in the sample (below detection limit) and no line indicates presence of toxin. The C line indicates that the sample fluid has sufficiently re-suspended and mobilized the antibody colour complex.
Source: Jellett Biotek Ltd., 2001.

4. Allow the test to run for 20 min, although most samples will provide a final result in about 10 min.
5. Read the result. Only one line (C – control) indicates that the test is positive for the presence of PSP toxins, nominally at or above the detection limit for the AOAC mouse bioassay (*c.* 400 µg STXeq kg^{-1} shellfish tissue). The development of two lines (C and T–toxin) indicates that the shellfish sample contains little toxin (<400 µg STXeq kg^{-1}) and would pass regulatory controls.
6. T lines of various intensities may occur due to different toxin analogues that can occur in the shellfish samples. For additional security, a T line of ≤50% of the C line intensity should be considered as a positive sample. T and C lines of equal intensity indicate a negative sample.
7. If no C line appears, the test must be considered invalid and performed again on another cassette. If the C line fails to appear again, there is something in the extract interfering with the reaction in the test kit. An alternative testing methodology must be used for these extracts. See MIST Alert™ instructions for detailed interpretation of C and T lines.

12.2.1.1.2 *Plankton samples*

The following procedure is for the assay of PSP toxins from plankton samples of laboratory cultures and field samples (Silva *et al.*, 2001, Cembella *et al.*, 2001*a*) using the MIST Alert™ with phytoplankton buffer:

1. Place 50 µl of phytoplankton extract prepared by the method of Appendix 12.1 into a 1 ml microcentrifuge vial containing 200 µl of phytoplankton buffer.
2. Perform serial dilutions by transferring 50 µl of diluted phytoplankton extract into a second vial with 200 µl phytoplankton buffer, mix well (10×) using same pipette tip, then transfer 50 µl into a third vial. Repeat until all desired dilutions are completed.
3. Open MIST Alert™ cassettes and label.

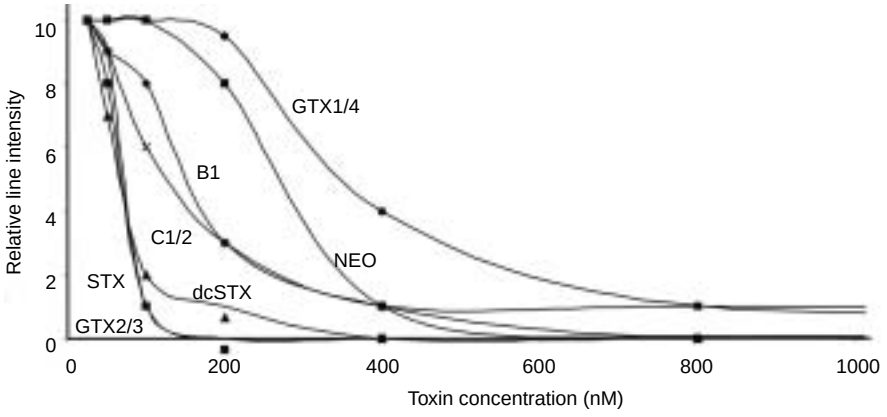

Figure 12.3
Plots of test line intensities of PSP toxins from dilution series measurements with the MIST Alert™ test.
Source: Laycock *et al.* (2001).

4. Pipette 100 µl of each dilution into each cassette well and wait at least 10 min for sample to diffuse before identifying the presence of either one or two lines.

When a sample contains PSP toxins, the MIST Alert™ will show only the C (control) line and the T (toxin) line will be absent or very faint. A negative sample that contains no PSP toxins or very low levels will show both lines. If a permanent record is to be kept, cassettes can be archived or optically scanned after labelling (date/location/origin/content). *[N.B.: With some plankton samples that contain high numbers of zooplankton or early developmental stages of invertebrates, an invalid response (no C line) may result. In this case, samples should be size-fractionated to remove organisms >100 µm prior to preparation for testing.]*

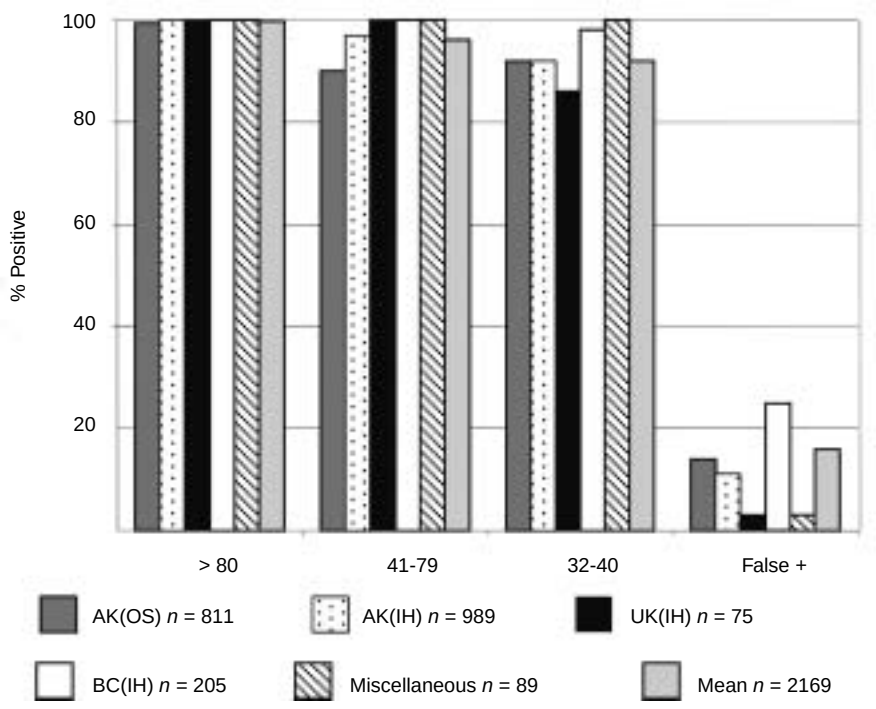

Figure 12.4
Shellfish tissue trials of MIST Alert™ for PSP versus the AOAC mouse bioassay in various regulatory laboratories; percentage positive values indicated for the MIST Alert™ are with reference to corresponding mouse bioassay toxicities (µg STXeq 100 g^{-1} shellfish soft tissues) in various ranges for the same samples. AK, Alaska; UK, United Kingdom; BC, British Columbia; IH, 'in house', i.e. laboratory; OS, on site.
Source: adapted from Jellett *et al*. (2002).

12.2.2 Immunoassays for ASP toxins (domoic acid analogues)

A number of immunodiagnostic procedures for the detection of domoic acid (DA) associated with anmesic shellfish poisoning (ASP) have been reported. Rabbit polyclonal antibodies have been used to develop radioimmunoassays (RIA) and ELISA methods to detect DA in mammalian serum and urine (Newsome *et al*., 1991). The RIA is rather complex to perform, and while suitable for use as a clinical research assay, is not amenable for routine assay of shellfish. An ELISA method using the same antibodies gave inconsistent results with various serum dilutions. Neither detection system has been subjected to the collaborative testing required for an approved routine technique. Smith and Kitts (1995) assayed shellfish tissue, and their ELISA results correlated well with HPLC values, but were around 9% higher, perhaps because the ELISA recognized DA isomers not detected by the analytical method. Unfortunately, this assay relies on the serum from a single mouse and will thus have limited use in shellfish testing. More recently, novel hapten chemistries have been used to raise antisera in sheep, and immunoassays using these highly specific and sensitive antisera have been established (Garthwaite *et al*., 1998*b*). These ELISAs, which can detect DA at more than 500 times below the MPL, have been employed to test both shellfish and seawater, and were used in conjunction with

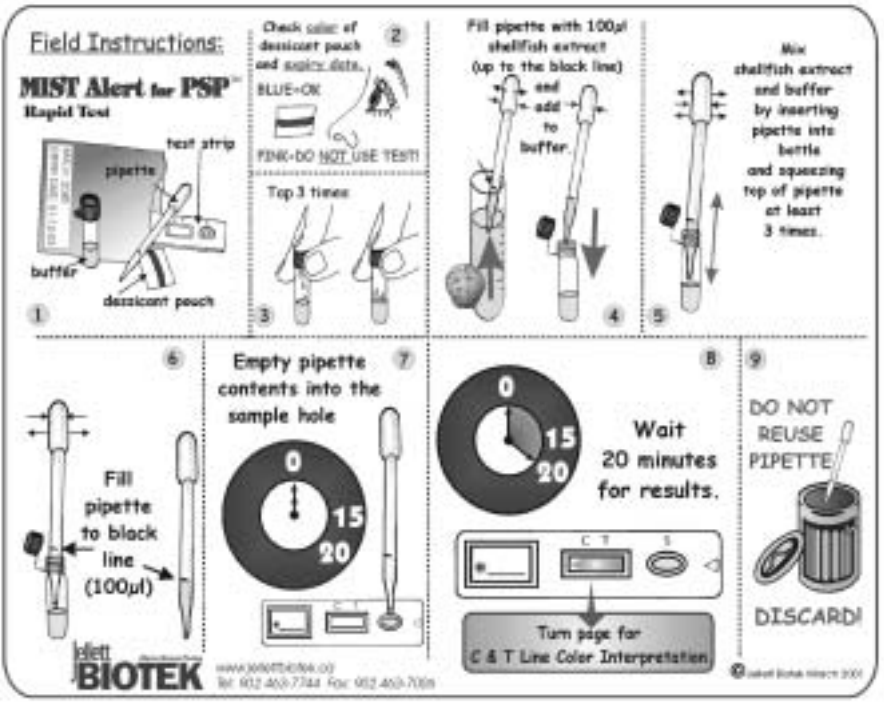

Figure 12.5
Instructions for the application of the MIST Alert™ for PSP for shellfish samples prepared in the field or in rudimentary laboratories.
Source: Jellett Biotek Ltd., 2001.

rRNA-targeted probes to demonstrate DA production by *Pseudo-nitzschia fraudulenta* (Rhodes *et al*., 1998). This assay has recently confirmed the presence of DA in Chilean shellfish (Garthwaite *et al*., unpublished data), and validation studies on shellfish are under way in Chile, New Zealand and the UK.

A simple alternative configuration for the detection of DA in shellfish and plankton matrices is the MIST Alert™ for ASP toxins (Jellett Biotek, Dartmouth, Canada), an immunochromatographic assay based on the same platform as previously described (Section 12.2.1.2) for PSP toxins. The antibodies for the MIST Alert™ for ASP toxins are produced by AgResearch (Hamilton, New Zealand) and have been well characterized by ELISA (Garthwaite *et al*., 1998*b*). The ASP toxin test kit works with either aqueous acidic or methanolic extracts, and thus can be applied to shellfish samples prepared for HPLC or LC-MS analysis (see details of extraction protocols in Chapter 9, Appendices) and to extracts prepared for the PSP mouse bioassay (AOAC, 2000).

The detection limit of the MIST Alert™ for ASP toxins is about 8–10 mg DA kg^{-1} for methanolic extracts, and about 2 mg DA kg^{-1} for hot aqueous acidic extracts (AOAC, 2000) – well below the 20 mg kg^{-1} regulatory limit. This test has also been recently applied with success to the detection of ASP toxins in samples from toxigenic cultures of *Pseudo-nitzschia multiseries* and natural plankton assemblages containing toxic diatoms (Cembella *et al*., 2001*b*). The nominal detection limit is 2–10 ng on the test strip.

12.2.3 Immunoassays for DSP toxins (okadaic acid analogues)

Several immunodiagnostic methods are available for the detection of DSP toxins, configured as either RIA (Levine *et al*., 1988) or ELISA tests (Chin *et al*., 1995; Park, 1995; Uda *et al*., 1989). Commercial sources for DSP toxin test kits and constituent antibodies are presented in Appendix 12.2. All these assays incorporate antibodies prepared against a single diarrhoeagenic agent, okadaic acid (OA), but they have some cross-reactivity against dinophysistoxin (DTX) analogues. The competitive binding [^{3}H]-labelling RIA procedure for OA detection (Levine *et al*.,1988) is highly sensitive (detection limit 0.2 pmol OA) and shows no competitive inhibition against various unrelated aquatic biotoxins, including maitotoxin (MTX), aplysiatoxin, palytoxin, brevetoxin B and lyngbyatoxin A. Yet this assay is somewhat time-consuming and complex for use as a routine method.

Test kits for DSP toxins have been produced for commercial distribution by SCETI Laboratories, Tokyo, Japan (DSP-Check), and Rougier Bio-Tech, Montreal, Canada. Unfortunately, the latter ELISA test kit is no longer in commercial production, and stocks of the antibody may be limited to that already distributed to research laboratories. These tests have been successfully used for the detection of OA and DTX toxins in shellfish tissues, specifically hepatopancreas (Chin *et al*., 1995; Carmody *et al*., 1995; Tubaro *et al*., 1992; Morton and Tindall, 1996). The assays have poorer sensitivity, however, when applied to whole shellfish tissues, and have only limited cross-reactivity with some of the DTX toxins, notably DTX2 and DTX3. Quantitation of DTX2 requires a specific assay, whereas DTX3 may be detected by any assay that cross-reacts with DTX1 by simply including a hydrolysis step to convert DTX3 to DTX1 (e.g. Suzuki *et al*., 1998).

The DSP-Check kit, which is now distributed in Europe by R-Biopharm GmBH, Darmstadt, Germany, has been widely used throughout the world for screen-

ing DSP toxins (OA and DTX1) at a claimed detection limit of 20 µg kg^{-1}. The monoclonal antibody in the DSP-Check test kit cross-reacts with DTX1 at a level comparable to OA. The pectenotoxins and yessotoxins, which are structurally unrelated, but included within the DSP group for regulatory purposes, are unreactive (Usagawa *et al*., 1989). Antibodies against yessotoxin and pectenotoxins have been raised at AgResearch, New Zealand (see Garthwaite *et al*., 2001). Both ELISA and affinity matrices are being developed which will allow ELISA methods to detect all toxins of the extended DSP group.

The Rougier Bio-Tech ELISA test kit utilizes an anti-OA monoclonal antibody and an anti-idiotypic antibody that competes with OA for binding sites on the anti-OA antibody (Shestowsky *et al*., 1993). The antibody in this kit exhibits a much higher sensitivity (10- to 20-fold) for OA than either DTX1 or DTX2, but certain methyl-, diol-, alcohol- and sulfated derivatives of OA are also bound by the antibody (Lawrence *et al*., 1998). The antibody does not cross-react with brevetoxins or DTX3. The test kit has undergone extensive comparison with analytical methods for DSP toxins (e.g. HPLC, LC-MS) and was found to be reliable for OA quantitation in both mussel extracts and phytoplankton (Chin *et al*., 1995).

12.2.3.1 *Anti-idiotypic antibody assay (Rougier Bio-Tech)*

The following protocol is for the use of the Rougier Bio-Tech antibody:

Reagents

- extraction solvent: 80% aqueous methanol;
- dilution solvent: 40% methanol in TRIS-buffered saline (TBS);
- monoclonal antibodies (MAb)
  anti-OA 6/50 MAb (100 ng ml^{-1}) in 40% methanol
  anti-OA anti-idiotypic 1/59 Ab (5 µg ml^{-1}) in 0.05 M Na citrate buffer, pH 9.6, produced according to Shestowsky *et al*., 1993;
- ELISA wash solution 0.05% Tween 20 in TBS (v/v);
- blocking agent: 1% whole-milk powder in TBS;
- enzyme solution: peroxidase-conjugated anti-mouse IgG Fc fragment-specific antiserum (Sigma Chemical Co., St Louis, Mo., USA) in 1% milk in TBS;
- colour reagent: 0.03% H_2O_2 in 0.1% o-phenylenediamine (OPD) (Sigma) and 0.1 M Na citrate buffer (pH 7.0);
- stop reagent: 1.5 M H_2SO_4;
- OA standard solutions: 0–1 µg ml^{-1} in 40% methanol.

Procedure

1. *Plate preparation:* Coat microtitre plates (e.g. Immulon I, Dynatech Laboratories, Chantilly, Va., USA) overnight at 4°C with anti-idiotypic 1/59 IgG (5 µg ml^{-1}) in 0.05 M Na_2CO_3-$NaHCO_3$ buffer, pH 9.6.
2. Wash off unbound antibody with wash solution.
3. Saturate plate with blocking agent to block any remaining sites. Incubate 1 h at 37°C. Wash plate.
4. Add 50 µl of standard or sample followed by 50 µl 6/50 IgG. Incubate 1 h at 37°C. Wash plate.
5. Add 100 µl enzyme solution to each well. Incubate 1 hour at 37°C. Wash plate.
6. Add 100 µl colour reagent. Cover plate. Incubate 30 min at room temperature.
7. Add 50 µl stop reagent.
8. Measure absorbance at 492 nm on a microplate reader.

9. Data analysis. Correlate concentration and optical density using linear regression analysis.

[N.B.: This is only an example of an indirect competitive ELISA, and this method may be adapted for other reagents by replacing the idiotypic antibody with protein-toxin conjugate.]

12.2.4 Immunoassays for CFP (ciguatera toxins) and NSP (brevetoxins)

Initial efforts to develop an immunodiagnostic method for ciguatoxin (CTX) detection in fish tissues were based on ^{125}iodine-labelled antibodies produced from sheep serum and incorporated into a direct RIA test (Hokama *et al*., 1977). This RIA technique was evaluated as a potential screening technique for ciguatoxic fish and was found to detect CTX in 93% of clinically documented ciguatera cases (Kimura *et al*., 1982). When configured as an EIA test, the results were similarly encouraging for screening of CTX in fish tissues, and competitive inhibition by a variety of related polyether toxins, including OA, MTX and brevetoxins (PbTx), was indicated (Hokama *et al*., 1984). The EIA technique using sheep anti-CTX antibody was eventually incorporated into a simple 'poke stick' test for the detection of polyether toxins in intact fish flesh (Hokama *et al*., 1987). This test was the subject of an AOAC inter-calibration study during 1999 and is under consideration as a first action screen. Other work on sheep immunoglobulins (Ig) (Berger and Berger, 1979) and on putative CTX antibodies prepared from rabbit serum and mouse ascites (Chanteau *et al*., 1981), involving enzyme-coupling reactions as a detection system (ELISA), failed to confirm antibody activity that correlated with ciguatoxicity. A direct RIA using Ig purified from mouse ascitic fluid (Parc *et al*., 1979) also revealed severe deficiencies in using polyclonal sera to detect CTX because of non-specific physico-chemical interference with antigen-antibody complexation. To overcome specificity problems, antibodies have recently been produced against the JKLM ring fragment of CTX. These antibodies have high specificity but low sensitivity for CTX 1B (IC_{50} = 2 µM), and have yet to be incorporated into a kit format (Pauillac *et al*., 2000).

The commercial Cigua-check™ immunodiagnostic test for CTX incorporates solid support immunobeads and is based on monoclonal antibodies that bind to the polyether skeleton of CTX and OA (Park, 1995). This allows for the screening of OA down to a detection limit of 50 ng kg^{-1} fish tissue. The lack of CTX standards has hampered the determination of relative cross-reactivity with various derivatives; therefore, the Cigua-check™ test can only be used as a general screening method to select samples for further analysis. The rate of false-positive responses has not yet been determined. Detailed descriptions of assay and chemical analytical methods for CTX are presented in Chapter 10, therefore the following assay details will be provided only for the detection of brevetoxins.

In the study of brevetoxins (PbTxs) associated with neurotoxic shellfish poisoning (NSP), antibodies have been raised against these lipid soluble toxins by a number of investigators, and immunoassays have been developed for research. Of the ten known PbTxs, the most common – brevetoxin B group toxins, PbTx2 and PbTx3 – have received the most study. Brevetoxin-specific antibodies were first developed in the early 1990s (Baden *et al*., 1984; Trainer and Baden, 1991; Levine and Shimizu, 1992; Poli *et al*., 1995). These antibodies were used for the development of immunoassays in a variety of formats: (i) RIA (see Sec-

tion 12.2.4.2); (ii) cell-based ELISA (see Section 12.2.4.1) (Baden *et al*., 1995); and (iii) signal-enhanced ELISA (Garthwaite *et al*., 1996) employing a biotinyl tyramide-horseradish peroxidase conjugate to improve assay sensitivity. The latter ELISA has been used for the detection of brevetoxins below 0.5 ng ml^{-1} in the culture medium of a number of *Karenia* (*Gymnodinium*) isolates (Garthwaite *et al*., 1998*a*). There are no validated, commercially available kits for NSP toxin immunoassay; however, antibody reagents may be purchased from the University of North Carolina at Wilmington, USA (see Appendix 12.2).

Studies investigating the epitope binding sites of naturally occurring and synthetic PbTx derivatives with two different anti-PbTx sera indicated that single antibody assays may not be adequate for detecting toxin metabolites (Poli *et al*., 1995). Tests are being developed to utilize multiple antibodies specifically designed for recognition of different regions of the polyether ladder (Melinek *et al*., 1994). Poli and co-workers have used RIA and a receptor binding assay to study extracts of shellfish and urine from patients diagnosed as suffering from NSP after eating shellfish (Poli *et al*., 2000). Affinity-purification of the shellfish extracts using specific anti-PbTx antibodies yielded four major peaks of activity. One peak was identified by LC-MS to be PbTx3, while the other peaks consisted of compounds of higher mass, suggestive of conjugated metabolites. Both RIA and receptor binding assay recognized the material; however, they quantified differently. This finding suggests that these metabolites react differently in the two assays, a result that may have important implications for seafood safety and regulation. The authors suggest that these metabolites may well be the true cause of NSP, and should be taken into account during regulatory testing. For example, monitoring for the usual brevetoxin mass/charge (*m/z*) ratios by LC-MS (see Chapter 8) would miss these compounds.

12.2.4.1 ELISA for brevetoxins

Immunoassays for NSP toxins are currently available only for research; however, commercialization is likely in the near future. To date, quantitation using ELISA test kits has not been fully demonstrated for fish or shellfish tissue samples and it is therefore recommended that this test procedure only be used for dinoflagellate extracts, prepared according to the procedure in Appendix 12.1.

Reagents

- Phosphate buffered saline (PBS)-Blotto, pH 7.2
  10 mM Na_2HPO_4
  0.15 M NaCl
  40 g l^{-1} non-fat dried milk (Blotto) [alternative: 0.25% bovine serum albumin (BSA)]; 0.01% sodium azide;
- ELISA wash buffer, pH 7.2, 0.5 M NaCl; 20 mM Na_2HPO_4;
- bicarbonate buffer, pH 9.6, 0.1 M $NaHCO_3$;
- horseradish peroxidase (HRP) substrate;
  20 ml 0.1 M Na citrate, pH 4.2
  1 ml 40 mM ABTS (2,2'-azino-bis-2-ethylbenzthiazoline-6-sulfonic acid)
  20 µl 30% H_2O_2
- alkaline phosphatase (AP) substrate
  100 µl BCIP (5-bromo-4-chloro-3-indolyl phosphate) (50 mg ml^{-1} stock in dimethyl formamide, DMF); store at 4°C

200 µl NBT (nitro-blue tetrazolium) (200 mg in 2 ml DMF, 2 ml distilled H_2O; store at 4°C)
30 ml TRIS buffer: 0.1 M TRIS base, pH 9.5; 0.1 M NaCl; 50 mM $MgCl_2$;

- SDS (sodium dodecyl sulfate), 10%;
- goat anti-PbTx antibody purification for this ELISA is the same as that described for the RIA in this section;
- rabbit anti-goat serum linked to HRP (Accurate Chemical Corp., Westbury, N.Y., USA, or from HABLAB Reagents, UNC, Wilmington, NC, USA, see Appendix 12.2).

Procedure

This assay protocol follows a four-step procedure illustrated in Trainer and Baden (1991):

1. Adsorption of sample to microtitre plate.
   a) Add serial dilutions of sample (100 µL) to 96-well microtitre plates and allow to equilibrate overnight at 4°C or for 2 h at room temperature in PBS buffer. Cover plates with plastic wrap.
   b) Rinse plate thrice with 200 µl ELISA wash buffer in each well.
2. Non-specific blocking of additional sites.
   Block wells 1 h with 100 µl PBS-Blotto. Cover plate with plastic wrap. Other acceptable blockers are 1% pre-immune serum and 1% gelatin (Knox, unflavoured). Rinse as in step 1b.
3. Specific binding of primary and secondary antibodies to toxin.
   a) Incubate >1 hour with primary goat antibody diluted in PBS-Blotto (100 µl in each well). Cover plate with plastic wrap. Rinse as in step 1b.
   b) Incubate with diluted secondary antibody (peroxidase-conjugated anti-goat IgG at a 1:1,000 dilution) in PBS-Blotto for at least 1 h (100 µl per well). Cover plate with plastic wrap. Rinse as in step 1b. Suggested dilution factors are 1:500–1:4,000 for HRP-linked antibodies and 1:2,000–1:5,000 for AP-linked antibodies. Optimal dilutions can be determined using a chequerboard assay.
4. Colour development and measurement.
   a) Add 100 µl of appropriate enzyme substrate into each well. Develop for about 15 min in the dark. HRP antibody is visualized with ABTS solution.
   b) Stop reaction after about 30 min by adding 25 µl of 10% SDS per well.
   c) Measure absorbance at 405 nm.
5. For data analysis, correlate concentration and optical density using linear regression.

[Notes: All reaction rates can be increased by increasing incubation temperatures. Blocker (PBS-Blotto) should only block non-specific binding. Too high a concentration of blocker may also block specific interaction. Washes should use a greater volume of buffer in each well than the previous incubation step. Plate should be developed in the dark as all dyes used are light sensitive.]

12.2.4.2 *Radioimmmunoassay for brevetoxins*

Antibody preparation and purification

Antigens are constructed using keyhole limpet haemocyanin (KLH) as a carrier protein employing procedures outlined in Baden *et al*. (1984), Trainer and Baden (1991), and Levine and Shimizu (1992). The following procedures for antibody

preparation and purification are applicable for serum processing after bleeding the animal.

Ammonium sulfate precipitation

1. Dilute plasma from bleeds with 0.1 volumes of saturated neutral ammonium sulfate solution, refrigerate overnight, and centrifuge at $3{,}000 \times g$ for 30 min.
2. Save the supernatant solution and discard the pellet. While stirring gently, slowly add more saturated ammonium sulfate solution to the supernatant, to bring the final ammonium sulphate concentration to 50% saturation. This results in an antibody containing precipitate that is harvested by centrifugation at $5{,}000 \times g$ for 1 hour.
3. Re-dissolve the pellet in 0.3 volumes of PBS, pH 7.4, and dialyse against three changes of PBS overnight, then freeze at -20°C until needed.

Protein G purification

- Wash buffer 0.01 M Na_2HPO_4;
- 0.15 M NaCl;
- 0.01 M EDTA, pH 7.0;
- Elution buffer 0.5 M acetic acid, pH 3.0.

1. Use a pump connected to a protein G column (Genex GammaBind Plus) for this step. Equilibrate the column with wash buffer and load the dialysed ammonium sulfate-precipitated antibody.
2. Wash the adsorbed IgG with 10 column volumes of wash buffer.
3. Elute the antibody with approximately one column volume of elution buffer and immediately neutralize with 1 M TRIS base.
4. Dialyse eluted IgG at 4°C against three changes of PBS overnight, and lyophilize for storage at -20°C until needed.

Brevetoxin affinity chromatography

1. Synthesize PbTx3-succinate via the method given in Baden *et al*. (1984).
2. Wash 5 g aminohexyl-sepharose with 3×50 ml of dH_2O, followed by 3×50 ml rinses with 50% pyridine. Add 5 ml of the resulting slurry to 300 µmol EDC in 0.5 ml 50% pyridine. Swirl the mixture for 2 hours at room temperature.
3. Add 9.9 mg PbTx3-succinate in 1 ml 50% pyridine, dH_2O, and PBS, pH 7.4 *(no azide!*). Store at 4°C.

Antibody purification

1. Wash the affinity column with 10 column volumes each of: (a) 10 mM TRIS, pH 7.5; (b) 10 mM glycine, pH 2.5; (c) 10 mM TRIS, pH 8.8; and (d) 10 mM TRIS, pH 7.5.
2. Load the protein G-purified IgG on to the column in 10 mM TRIS, pH 7.5. Recirculate thrice through the column. Rinse with 10 mM TRIS, pH 7.5 until no more protein is eluted (monitored by Bradford protein assay).
3. Elute the sample using 10 column volumes of 0.1 M glycine, pH 2.5 (3 M NaCl in PBS, pH 7.4 has also worked well). Neutralize eluted IgG with 1 M TRIS base, dialyse against three changes of PBS overnight and store in 1 ml aliquots at -70°C.
4. Regenerate the column by repeating step 1.

Reagents

- phosphate buffered saline (PBS), pH 7.4. For 1 l stock solution:
  1.392 g K_2HPO_4;
  0.276 g NaH_2PO_4;
  8.770 g NaCl;
  1 g Na azide for a 0.1% solution for storage;
  Dissolve in 900 ml distilled H_2O and adjust pH to 7.4 using KOH. Bring volume to 1 l.
- Dextran-coated charcoal (100 ml):
  10 g neutral charcoal;
  0.25% Dextran in PBS [Note: The working mixture is a 1:10 dilution of this stock in *PBS.]* Keep on ice and stir constantly.
- Antibody solution: anti-PbTx antibody can be prepared following the references given here, or purchased (see Appendix 12.2).

Assay Procedure

1. Add 0.2 ml toxin or sample to 1.5 ml microcentrifuge tubes.
2. Add 0.2 ml [^{3}H]-PbTx3 to centrifuge tubes. Use a serial dilution in triplicate (10, 1, 0.5, 0.25, 0.125, 0.0625 nmol).
3. Add 0.2 ml PBS to centrifuge tubes. Also set up one triplicate sample with 10 µM cold PbTx3 instead of PBS for determination of non-specific binding.
4. Add 0.1 ml of antibody (1.5 mg protein).
5. Close tubes and invert several times to mix.
6. Incubate 1 hour on ice.
7. Add 0.5 ml dilute charcoal to each tube, then close, invert and mix. Incubate 2.5 min.
8. Centrifuge at maximum speed in the micro-centrifuge for 2–3 min.
9. Assay supernatant solution for radioactivity.
10. Analyse data.

[Note: Free toxin should be bound within the Dextran-charcoal matrix. Toxin bound to the antibody remains in the supernatant.]

12.3 Development and application of enzyme-inhibition assays

Functional *in vitro* enzymatic assays for phycotoxin detection are comparatively rare, primarily because most of these toxins do not inhibit enzymes. Nevertheless, Holmes (1991) successfully exploited the specific inhibition of protein phosphatase Type 1 (PP1) and Type 2A (PP2A) by certain DSP toxin analogues (OA and DTX1) in the development of a phosphatase radioassay using ^{32}P phosphorylase. Although this technique, which is coupled with toxin fractionation by liquid chromatography, is not in wide circulation as a regulatory tool, it has been used in screening the phosphatase inhibition activity of putatively phycotoxic compounds and partially purified extracts of phytoplankton and shellfish. When applied to the analysis of naturally contaminated mussel tissue, extracts of cultured *Prorocentrum lima*, and net tow material from natural phytoplankton assemblages, the assay revealed that the PP1/PP2A inhibition activity exceeded that which could be accounted for by total levels of OA and DTX1 – suggesting the presence of cryptic, but potentially diarrhoe-

agenic toxins (Luu *et al*., 1993). The enzyme inhibition assay is also useful for the detection of microcystins, a class of phycotoxins produced by certain cyanobacteria (see Chapter 11), and other toxins capable of inhibiting PP1.

This enzyme assay is based on the inhibition of PP1 by OA at a sensitivity as low as 0.1 pg OAeq kg^{-1} tissue (Baden *et al*., 1995). Additional research on the use of a mutant PP1 promises to improve the sensitivity of this test by an order of magnitude (Zhang *et al*., 1994). A useful version of this PPase inhibition assay, based on colourimetric detection, has been applied for the assay of DSP toxins in shellfish and plankton (Tubaro *et al*., 1996*b*), and further refinements have been recently incorporated (Della Loggia *et al*., 1999). A fluorescence-detection version of this assay has also been developed and successfully used for the detection of DSP toxins (Vieytes *et al*., 1997). Although the fluorimetric assay offers better sensitivity, and may be preferred in direct comparisons with the colourimetric version (Mountfort *et al*., 1999), it requires a fluorescence plate reader – a device not available in many laboratories.

12.3.1 Protein phosphatase assay

The following protocol for the colourimetric protein phosphatase inhibition assay using PP2a (96-well plate format) is modified from Tubaro *et al*. (1996*a*, 1996*b*) and is suitable for the detection of activity of either DSP toxins or microcystin-LR:

Materials and reagents

- TRIS-HCl, pH 8.4;
- $MgCl_2$;
- EDTA, disodium salt;
- dithiothreitol (DTT);
- dimethyl sulfoxide (DMSO);
- p-nitrophenol phosphate (pNPP);
- protein phosphatase 2A (PP2A), Upstate Biotechnology, Lake Placid, N.Y., USA, cat. #14–111;
- OA-certified standard, Insitute for Marine Biosciences, National Research Council (NRC), Halifax, Canada, cat. #OACS;
- OA-sodium salt, Alexis Biochemicals, San Diego, Calif., USA, cat. #350–11C100;
- reagent reservoir, Costar, Cambridge, Mass., USA, cat #4870;
- 96-well flat-bottom plate, Costar, cat. #3370;
- 1.2 ml microtitre dilution tube, United Lab Plastics, St Louis, Mo., USA, cat. #UP2080;
- 50 ml tube, United Lab Plastics, cat. #UP2009;
- 15 ml tube, United Lab Plastics, cat. #UP2023;
- pipettors: 1–20 µl, 20–200 µl, 100–1,000 µl; multichannel pipette: 50–300 µl;
- microplate reader with 405 nm filter;
- latex or nitrile gloves.

Basic preparation protocol

A. Buffer: (25 ml required for one plate):
   40 mM TRIS-HCl, pH 8.4;
   34 mM $MgCl_2$;

4 mM EDTA;
4 mM DTT; add *fresh daily* (100 µl per 25 ml from frozen stock solution: 1 M DTT in dH_2O).

B. Preparation of substrate: 140 mM pNPP (approximately 6 ml needed for 96 wells); 221 mg pNPP per 6 ml buffer.
C. Protein phosphatase: 0.2 units μl^{-1} PP2A (Upstate Biotechnology, Lake Placid, N.Y., USA); PP2A from other sources may have different activities (approximately 2.4 ml are needed for 48 wells, no PP2A is added to the other half of 96-well plate). Dilute 1:1600 in buffer (3.5 µl PP2A per 5.6 ml buffer) to prepare working solution for assay. Final concentration in each well containing PP2A is 0.025 units ml^{-1}.
D. Preparation of OA standards: OACS-1 should be used as primary standard (National Research Council, Canada). Make up working OA standards by serial dilution of the stock standard in buffer. *[N.B.: OACS-1 solution is 25.5 µg OA* μl^{1} *(=* 3.03×10^{-5} *M)]*. Prepare standards for the range 5×10^{-6} to 5×10^{-10} M. Standards are diluted 1/5 in the assay to give a standard curve range of 10^{-6} M to 10^{-10} M OA.
E. Dilute all samples with buffer or water to at least 1:1 (methanol will destroy PP2A). Samples may require further dilution to fall within the linear range of the standard curve.

Assay procedure

1. Prepare the buffer fresh daily as described in step A.
2. Dilute the PP2A, prepare the pNPP substrate, and prepare the OA standard curve as described in steps B, C and D.
3. Dilute the unknowns in dH_2O or buffer, as described in step E.
4. In duplicate, add samples, buffer, and PP2A to a flat bottom 96-well plate *in the following order:*
   a) add 50 µl of OA standards or unknowns to wells; add 50 µl buffer to the reference and blank wells.
   b) add 100 µl of buffer to each well.
   c) add 50 µl of diluted PP2A to each well *except the blank wells*; add 50 µl dilution buffer to the blank wells. *[N.B.: PP2A is added last because this enzyme can be destroyed by the methanol in the sample.]*
5. Add 50 µl of pNPP substrate to each well in rapid succession to start enzymatic reaction.
6. Incubate the plate at room temp for 60 min.
7. Measure the absorbance of the samples in the plate at 405 nm using the plate reader.
8. The relative OA concentration in the unknown samples can be calculated by generating a standard curve from the OA standards run with each plate.

12.4 Development and application of receptor assays

Receptor assays were initially developed to investigate the properties of ion-conducting channels, and to characterize the interaction of various ligands with their channel receptors. Despite the variations noted, many of the phycotoxins do share a common characteristic. The toxins responsible for PSP, ASP, NSP and CFP all exert

their first-order toxic effects by binding to a certain class of biological receptors and, except for ASP toxins, which target the glutamate receptor, these receptors are exclusively either voltage-dependent Na^+ or Ca^{++} channels. This highly specific interaction with naturally occurring receptors is the basis of the receptor assay approach to phycotoxin detection. In practical terms, any modifications to a toxin molecule that interfere with its binding to the receptor, and thus its detection by a receptor-based assay, would also compromise its ability to elicit a toxic response. Toxin detection is therefore based on its functional activity rather than on recognition of a structural component. Another important component of the toxin-receptor association is the high affinity of this interaction. The receptors that bind phycotoxins do so with an affinity that matches, or in some cases exceeds, that characterizing antibody-antigen binding. The affinity of a toxin for its receptor (binding constants in the nanomolar range) is usually directly proportional to its toxic potency *in vivo*; however, this depends on the uptake mechanism and the site of toxin action. For a mixture of toxic congeners, a receptor-based assay will normally yield a response representative of the integrated potencies of those toxins present.

In the case of STX, use of radio-receptor assays has provided a detailed description of toxin binding kinetics and estimates of Na^+ channel densities in excitable membranes. The first attempt to utilize a receptor binding approach for the detection of STX employed [^{3}H]-labelled STX in a competitive displacement assay, but was tested only on samples prepared in isotonic buffer and human plasma (Davio and Fontelo, 1984). The method was later modified by Vieytes *et al*. (1993) to increase the sample capacity through the use of microtitre plates, and it was shown to be capable of detecting PSP toxins in shellfish extracts. The STX radio-receptor binding assay is a competitive binding assay in which radio-labelled and unlabelled STX and/or its derivatives compete for a given number of available receptor sites in a rat brain membrane preparation. The percentage reduction in radio-labelled STX binding is directly proportional to the amount of unlabelled toxin present in either a certified reference standard or an unknown sample.

The STX radio-receptor binding assay has been further refined (Charleston Laboratory, U.S. National Ocean Service) to simplify the protocol and enhance the overall efficiency of the assay in formats employing either conventional (Doucette *et al*., 1997) or microplate (Powell and Doucette, 1999) scintillation counting. The assay has also been recently validated for use with 11–[^{3}H]-tetrodotoxin (TTX) as an alternative radio-ligand to tritiated STX (Doucette *et al*., 2000). This technique, described in this section in general terms (see Fig. 12.6), has been extensively validated for use with extracts from a variety of shellfish, and it has performed extremely well in comparisons with the AOAC mouse bioassay. Effective use of the assay in both laboratory and field studies of toxic dinoflagellates, as well as in the detection of PSP toxins in human fluids, has also been demonstrated (Powell and Doucette, 1999).

The assay is formatted for use with the Millipore MultiScreen™ Assay System (Fig. 12.6), and is performed in a microtitre plate with wells occluded by hydrophilic, low-protein binding Durapore® membranes, which facilitate the exchange of solutions by vacuum filtration on a manifold designed specifically for these membrane plates. To conduct an assay, a volume of [^{3}H]-STX is added to each pre-wetted well such that its final concentration approximates the dissociation constant, K_D (0.5–5 nM for STX). An equal volume of the reference standard or sample is then added, followed by about four volumes of rat brain membrane preparation. The

assay components are incubated, then rinsed with buffer to remove any unbound labelled or unlabelled toxin. For use with a conventional scintillation counter, the filters are transferred into scintillation vials using a dedicated punch apparatus (Millipore) that simultaneously excises one row of eight filters. The filters are allowed to stand overnight in liquid scintillant prior to determining the radioactivity retained on the membrane by standard liquid scintillation counting. By employing microplate scintillation counting, the microfiltration plate can be left intact, liquid scintillant added directly to the wells, and the entire plate counted in approximately 15 min. In both cases, the quantity of toxin in a sample is obtained from a binding competition curve, which is a semi-log plot of percentage total binding of the [^{3}H]-STX (B/B_{max}) against a range of unlabelled STX standard concentrations (Fig. 12.7).

The receptor assay response reflects the integrated toxic potency of all PSP toxins (or other site-1 Na^+ channel blockers; e.g. TTX) present in a sample, relative

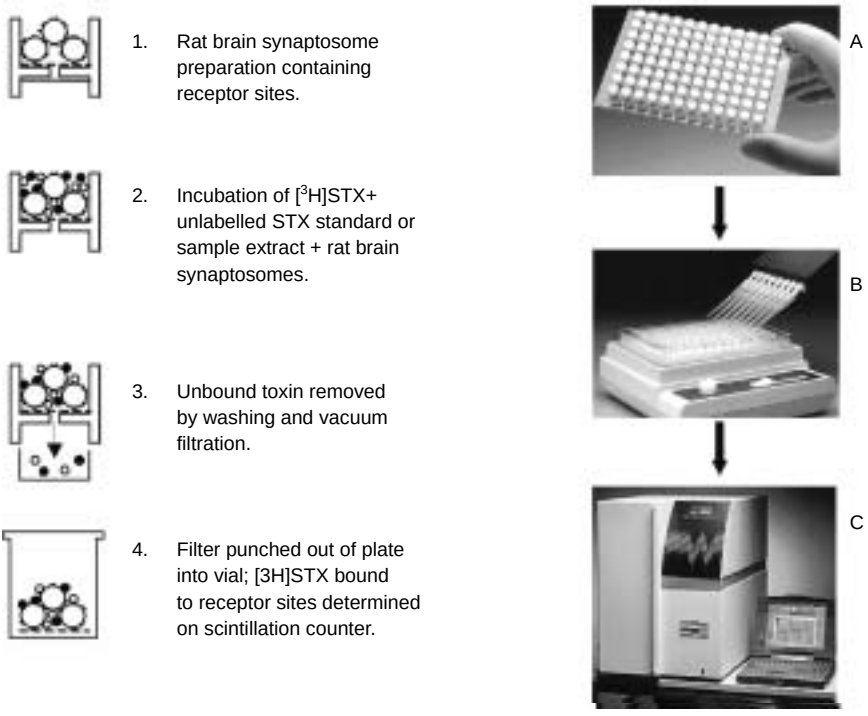

Figure 12.6
Schematic diagram of STX radio-receptor binding assay performed using the Millipore MultiScreen^{TM} Assay System. The view represents a cross-section of a microtitre filtration plate well, with dashed lines indicating the filter membrane. Solid circles show [^{3}H]-STX; open circles indicate unlabelled toxin in standard or sample extract (see text for detailed protocol). Also shown are photographs of A, 96-well filtration plate; B, washing and vacuum filtration of the plate; C, Wallac TriLux 1450 MicroBeta microplate scintillation counter.
Source: adapted from Cembella *et al.* (1995).

to purified STX; toxicity is thus expressed in STX equivalents (STXeq). The limit of detection for this assay is *c.* 4 ng STXeq ml^{-1} in the original sample extract, which is several orders of magnitude more sensitive than the mouse bioassay. The STX receptor binding assay can generate results highly consistent with those produced by the mouse assay. A detailed protocol, including the necessary equipment, supplies and reagents, is given in Section 12.4.1.

While structurally unrelated to the voltage-gated Na^+ channel, the novel transferrin molecule known as saxiphilin binds STX with an affinity in the low nanomolar range and thus equivalent to that of the Na^+ channel. However, unlike the Na^+ channel receptors, saxiphilin, a soluble, hydrophilic circulatory protein isolated from certain vertebrates and invertebrates, does not bind TTX and thus has the potential to discriminate between this toxin and STX analogues. Recently, Llwellyn and coworkers incorporated saxiphilin into a radio-receptor binding assay (Negri and Llewellyn, 1998) and ultimately transferred the method into a high throughput, micoplate format (Llewellyn *et al*., 1998). A comparison of the saxiphilin-based receptor assay with HPLC analysis of extracts from various marine species showed a high degree of correlation when the latter results were expressed as STXeq (Llewellyn *et al*., 1998), although the sample toxin profiles tested were restricted to

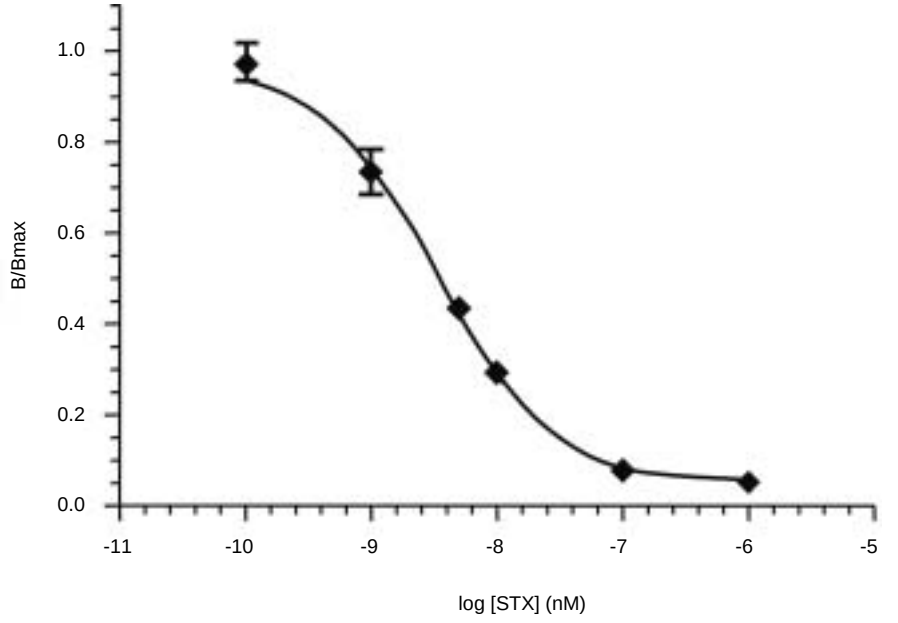

Figure 12.7
Compilation of six competitive binding curves performed on different days, demonstrating low inter-assay variability on the microplate scintillation counter. Percentage total binding of [^{3}H]-STX (B/Bmax) is plotted against the concentration of unlabelled STX. Error bars represent ± 1 S.D. The STX concentration that yields 50% total binding (i.e. IC_{50}) is *c.* 5 nM.

only STX and/or dcSTX. Assessment of saxiphilin binding affinity for multiple PSP toxin congeners showed similar values among STX, dcSTX, NEO and B1, but two to three orders of magnitude lower for the N-sulfocarbamoyl–11-hydroxysulfate toxin, C1 (Llewellyn *et al*., 1997). By comparison, the Na^+ channel receptor affinity for C toxins is within a factor of ten, relative to the highly potent carbamate derivatives (Doucette *et al*., 1997). Nevertheless, the saxiphilin-based receptor assay shows good promise as a robust technique for detecting PSP toxins in a wide range of sample matrices, especially when discrimination from TTX is required.

Receptor binding assays for PbTx and CTX are based on the interaction with site-5 on the voltage-sensitive Na^+ channel. A radioassay based on competitive binding with tritiated brevetoxin ([^{3}H]-PbTx3) is described in Section 12.4.3. As with the STX receptor assay, the extent of binding to the Na^+ channel is directly related to toxicity; therefore, the results are comparable to the mouse bioassay. The functional receptor binding assay has been compared with the RIA and fish bioassay for PbTx (Baden *et al*., 1988), as presented in Table 12.1, but the RIA for PbTx is now far more sensitive (ED_{50} < 1 nM; Poli *et al*., 1995). Work is progressing towards the use of isolated or reconstituted ion channels as a 'sensor' for detecting voltage-sensitive Na^+ channel toxins (Trainer *et al*., 1995; Fairey *et al*., 2001). These techniques promise to improve the sensitivity of the existing bioassay methods while retaining the ability to distinguish between toxin classes (e.g. Na^+ channel activators versus blockers). An advantage to employing cloned receptors is that the use of vertebrate animals is avoided, and ultimately the problem of obtaining a consistent source of receptors for various assays may be solved.

TABLE 12.1. Correlation of potency with radioimmunoassay and receptor assay. Ranges for RIA and receptor assay, and 95% confidence interval for the fish bioassay, are shown in parentheses.

Toxin	Toxin concentration (nM)		
	ED_{50} radioimmunoassay[a]	LD_{50} fish bioassay[b]	ED_{50} receptor assay[c]
PbTx1	93(90–130)	4.4 (3.8–5.0)	3.5 (3–5)
PbTx7	92 (90–120)	4.9 (2.2–10.7)	4.1 (4–7)
PbTx3	20 (16–25)	10.9 (7.2–22.0)	12.0 (10–15)
PbTx2	22 (18–29)	21.8 (17.2–35.0)	17.0 (15–18)

a. Average of three experiments; procedure from Baden *et al.* (1984).
b. Median lethal dose (n = 3): *Gambusia* fish assay, 20 ml water and 60 min assay time.
c. Average of three experiments.
Source: Baden *et al*. (1988)

A radio-receptor binding assay for DA, using a frog (*Rana pipiens*) brain membrane preparation, is based on competitive binding with [^{3}H]-kainic acid for the kainate/quisqualate glutamate receptor (Van Dolah *et al*., 1994, 1995). This sensitive method (IC_{50}: 0.89 ± 0.07 nM), which also employs the rapid, high-throughput microplate scintillation counting technology described previously, was recently modified to incorporate a cloned rat GLUR6 receptor and now includes pretreatment of samples with glutamate decarboxylase to remove endogenous glutamate that interferes with DA binding to its receptor (Van Dolah *et al*., 1997).

Preliminary testing of naturally contaminated shellfish reference material has demonstrated a close correspondence to calculated values. In addition, this assay has been used extensively to detect DA in tissues and fluids of marine mammals (Scholin *et al*., 2000), in fish (Lefebvre *et al*., 1999) and various invertebrates (Powell *et al*., 2001), as well as in laboratory (Pan *et al*., 2001) and field populations of toxic algae (Parsons *et al*., 1999).

12.4.1 Receptor binding assay for PSP toxins (saxitoxin analogues)

The receptor assay for PSP toxins is a competitive binding assay in which radio-labelled STX competes with unlabelled STX and/or its derivatives for a given number of available receptor sites in a rat brain membrane preparation. The percentage reduction in radio-labelled STX binding is directly proportional to the amount of unlabelled toxin present in either a certified reference standard or an unknown sample. Binding of [^{3}H]-STX can be determined either by conventional or microplate scintillation counting, and both methods are given in this section. Acidic, aqueous shellfish extracts are prepared for testing according to the AOAC (2000) method (see Chapter 13).

Reagents

- [^{3}H]-STX diacetate (Amersham, Arlington Heights, Ill., USA.; cat. #TRK 922);
- STX dihydrochloride Reference Standard (National Research Council, Halifax, Canada);
- 75 mM HEPES/140 mM NaCl buffer (35.75 g HEPES + 16.36 g NaCl in 2 l), pH 7.5;
- Rat brain membrane preparation (protocol follows in this section).

Supplies

Needed for both conventional and microplate scintillation counting

- 96-well microtitre filter plate with 0.65 µm Duropore membrane and type B glass fibre filter (Millipore, Bedford, Mass., USA.; cat. #MAFB NOB 50);
- MultiScreen vacuum manifold (Millipore; cat. #MAVM 096 01);
- 15 ml and 50 ml conical plastic centrifuge tubes;
- variable-volume micropipettors and disposable tips;
- eight-channel variable volume micropipette and disposable tips;
- mini-dilution tubes;
- reagent reservoirs;
- vortex mixer;
- vacuum source.

Needed only for conventional scintillation counting

- MultiScreen disposable punch tips (Millipore; cat. #MADP 196 10);
- MultiScreen punch kit-B for 4 ml vials (Millipore; cat. #MAPK 896 0B);
- liquid scintillation cocktail (e.g. Scintiverse, Fisher Scientific);
- scintillation vials (e.g. Wheaton omnivials; cat. #225402).

Needed only for microplate scintillation counting

- microplate carrier cassette (Perkin-Elmer Wallac, Inc., Gaithersburg, Md., USA, cat. #1450-105);
- sealing tape (Millipore, cat. #MATA HCL 00);

- liquid scintillant, e.g. Optiphase Supermix (Perkin-Elmer Wallac, Inc.; cat. #1200-439);
- microplate scintillation counter (TriLux 1450 Microbeta; Perkin-Elmer Wallac, Inc.).

Preparation of stock solutions and standards

1. *[^{3}H]-STX stock solution.* The following is an *example* for commercially available [^{3}H]-STX diacetate with information included on the specification sheet: specific activity 14.9 Ci mmol^{-1}, volume 200 µl, amount of radioactivity 50 µCi.
   50 µCi/(14.9 µCi nmol^{-1}) = 3.36 nmol; STX concentration = 3.36 nmol per 0.2 ml = 16.8 µM.
   The working stock concentration of [^{3}H]-STX is 13.3 nM and the final assay concentration is 2.2 nM; to make up enough 13.3 nM [^{3}H]-STX for one 96-well plate, add 3 µl [^{3}H]-STX to 3.8 ml of HEPES/NaCl buffer.
2. *Unlabelled STX solutions* (i.e. standard curve and inter-assay calibration standard). STX dihydrochloride Reference Standard is provided at a concentration of 67.2 µM. Perform the dilutions indicated in Table 12.2 to construct the standard curve. An inter-assay calibration standard containing 3.0×10^{-8} M STX standard (5.0×10^{-9} M STX in assay) made up in 3 x 10^{-3} M HCl and stored frozen in 100 µl aliquots should be thawed at the time of the assay and included in *each* analysis (duplicate wells) in order to confirm day-to-day performance.

TABLE 12.2. Preparation of the STX standard curve for the receptor assay

	Concentration of standard[a]	**Concentration in assay**
22.3 µl 67.2 µM STX + 228 µl HEPES/NaCl buffer	6.00×10^{-6}	1.0×10^{-6}
50 µl 6.00×10^{-6} solution + 450 µl buffer	6.00×10^{-7}	1.0×10^{-7}
50 µl 6.00×10^{-7} solution + 450 µl buffer	6.00×10^{-8}	1.0×10^{-8}
25 µl 6.00×10^{-7} solution + 475 µl buffer	3.00×10^{-8}	5.0×10^{-9}
50 µl 3.00×10^{-8} solution + 200 µl buffer	6.00×10^{-9}	1.0×10^{-9}
50 µl 6.00×10^{-9} solution + 450 µl buffer	6.00×10^{-10}	1.0×10^{-10}
Reference	Buffer only	0

a. Standards are diluted 1:6 in the assay.

3. *Rat brain membrane preparation*. Dilute the stock rat brain membrane preparation in ice-cold 75 mM HEPES/140 mM NaCl (pH 7.5) to yield a final protein concentration of about 0.5 mg ml^{-1}.

Rat brain membrane preparation for PSP toxins

1. Purchase 20 brains of six-week-old, male Holtzman rats (Harlan Bioproducts, Indianapolis, Ind., USA; cat. #BT 403) or extract from freshly killed animals.
2. Remove medulla and discard; place all cerebral cortices in a Petri dish containing 20 mM TRIS/140 mM NaCl (pH 7.1) on ice. 20 mM TRIS/140 mM NaCl: 2.1 g TRIS; 8.18 g NaCl.

3. 1 ml 1.0 M PMSF* (phenylmethylsulfonyl fluoride; Sigma, cat. #P7626); adjust pH to 7.1; bring to a final volume of 1 l with ddH_2O. *dissolve 0.174 g PMSF in 1 ml isopropanol; larger volumes may be made, dispensed into 1 ml aliquots, and stored at -20°C.
4. Place two cortices and 25 ml of 20 mM TRIS/140 mM NaCl (pH 7.1) into glass homogenizer tube and homogenize using Teflon probe at 385 rpm. It is *very important* to keep each preparation *on ice* during the homogenization step. Pour homogenized tissue into 250 ml beaker kept on ice and repeat this procedure for remaining cortices.
5. Place pooled homogenate in ultracentrifuge tube(s) always keeping preparation *on ice*, and centrifuge at 54,000 × *g* for 15 min at 4°C.
6. Aspirate supernatant and re-suspend pellet(s) with 10 ml ice-cold TRIS/NaCl buffer per brain (i.e. 200 ml total volume) in a beaker. Keep on ice.
7. Homogenize pellet/buffer suspension using a probe sonicator at 385 rpm for 20 s, holding preparation *on ice* at all times.
8. Dispense membrane preparation into 2 ml aliquots and freeze immediately at -80°C; this preparation is stable for at least six months. Perform assay to determine protein concentration and establish appropriate dilution to yield *c.* 0.5 mg protein per millilitre in the receptor assay.

Assay Procedure

For both conventional and microplate scintillation counting

1. Add the following to each of 96 wells *in the order given*:
   - 35 µl ice-cold 75 mM HEPES/140 mM NaCl (pH 7.5) to pre-wet filters;
   - 35 µl [^{3}H]-STX;
   - 35 µl STX standard or sample;
   - 105 µl membrane preparation (at a dilution yielding *c.* 0.5 mg protein per millilitre 'in assay').

[N.B.: All standards and samples should be run in duplicate; arrange samples vertically in the plate to simplify organization after filters are punched into vials.]

2. Incubate for 1 hour at 4°C.
3. Filter the entire plate on top of MultiScreen vacuum manifold.
4. Rinse each well once with 200 µl of ice-cold HEPES buffer using multichannel pipette.

For conventional scintillation counting

5a. Remove the plastic bottom of the 96-well filter plate, blot bottom of plate once on absorbent towel, set plate into MultiScreen punch, and place disposable punch tips on top of plate.

6a. Punch wells into vials pre-filled with about 4 ml liquid scintillant. Put caps on vials and vortex each sample.

7a. After allowing vials to sit at room temperature for 6 hours, count in standard liquid-scintillation counter.

8a. Data analysis.

For microplate scintillation counting

5b. Remove the plastic bottom of the 96-well filter plate, blot bottom of plate once on absorbent towel, and place microplate into carrier cassette.

6b. Seal cassette bottom with sealing tape, add 25 µl Optiphase Supermix liquid scintillant to each well, and seal the top of the plate with sealing tape.

7b. After allowing vials to stand at room temperature for 30 min, count on microplate scintillation counter.
8b. Data analysis.

Sample value calculations

The concentration of PSP toxins (in nanomolar STXeq) in samples is determined from a competitive binding curve. If the data are graphed as a semi-logarithmic plot of percentage total binding of [^{3}H]-STX against the concentration of unlabelled STX reference standards, the curve is typically sigmoidal (Fig. 12.7). Thus, it is *essential* to transform the percentage total binding data using a logit transformation, or similar procedure, and graph the resulting values on a linear *y*-axis scale against a logarithmic *x*-axis scale of unlabelled STX concentration.The logit transformation is performed as follows: logit (y) = ln [(B/B_{max})/(1 – B/B_{max})], where B/B_{max} = percentage total binding (i.e. total counts for a given unlabelled STX standard/total counts for reference containing no unlabelled STX). Once the data points are plotted, a line of best fit is generated using a simple linear regression. The STX concentrations for the sample unknowns are then obtained by solving the regression equation for x using the logit transformation of B/B_{max} for sample (y). *[N.B.: the displacement curve can also be generated by a computer program such as LIGAND (McPherson, 1985), or others, such as the four parameter logistic transform that will also calculate STX equivalent concentrations for sample unknowns.]* That part of the curve falling between 30% and 70% total binding provides the most accurate determination of STX concentrations in unknown samples. Samples yielding percentage total binding values outside of this range should be diluted appropriately and re-run. Final concentrations for unknown samples are determined by multiplying the calculated 'in assay' value by the assay dilution factor (i.e. 6.0) and any sample dilution factors. The optimal slope of the standard curve should be 1.0. While this is often not achieved, it is *important* that the slope of the regression line fall between 0.8 and 1.2. Otherwise, the sample values obtained will be of questionable reliability.

12.4.2 Receptor binding assay for ASP toxins (domoic acid analogues)

A portion of the DA receptor binding assay protocol, including the data analysis, is identical to that described above for the STX receptor assay, except for the use of a glutamate receptor preparation and the need to eliminate interference from endogenous glutamate in samples by pre-digestion with the enzyme glutamate decarboxylase (GAD). Thus, steps common between both assays will be referred to the STX protocol. Note that one of the advantages of the receptor-based approach to phycotoxin assays is that once a laboratory is set up to perform such assays, the only significant change required to test for a different toxin is substitution of the appropriate receptor preparation and radio-labelled toxin or analogue. Finally, this assay (but *not* the STX receptor assay, due to loss of the exchangeable tritium label during heating) can also be performed more economically using a 96-place GF/C glass fibre filter mat (Perkin-Elmer Wallac Filtermat A) in combination with a solid wax scintillant (Perkin-Elmer Wallac Meltilex) as described by Van Dolah *et al*. (1997).

Reagents

- [^{3}H]-kainic acid (Perkin-Elmer Life Science Products, Boston, Mass., USA; cat. #NET875);

- DACS-1D domoic acid reference standard (National Research Council, Halifax, Canada);
- 50 mM TRIS, pH 7.4;
- frog brain membrane preparation (protocol follows in this section).

Supplies

See list of supplies for STX receptor assay (Section 12.4.1); either conventional or microplate scintillation counting can be used.

Preparation of stock solutions and standards

1. [^{3}H]-kainic acid; specific activity 30–60 Ci mmol^{-1}, 1 mCi ml^{-1}; store at -20°C. Prepare 30 nM working stock solution daily: 7 µl [^{3}H]-kainic acid in 4.0 ml 50 mM TRIS, pH 7.4 (exact volumes will depend on the specific activity of the isotope).
2. Unlabelled DA solutions (i.e. standard curve and inter-assay calibration standard). DACS-1 Reference Standard supplied as a 87.7 µg ml^{-1} solution in 1:9 acetonitrile:H_2O. Prepare 57.2 µM working stock solution in 1:9 acetonitrile:H_2O: 101.4 µl DACS-1D in 398.6 µl 1:9 acetonitrile:H_2O; store refrigerated. Perform the dilutions indicated in Table 12.3 to construct the standard curve. An inter-assay calibration standard containing 2.86×10^{-6} M DA is made up by diluting 50 µl of DACS-1D working stock (57.2 µM) in 950 µl 1:9 acetonitrile:H_2O, dispensed in 100 µl aliquots, and stored refrigerated until use. This calibration standard is then diluted 1:100 in GAD buffer (see following procedure) and run in *each* analysis (duplicate wells) in order to confirm day-to-day performance. The 'in assay' concentration of the calibration standard is 4.8×10^{-9} M.

TABLE 12.3. Preparation of the domoic acid standard curve for the receptor assay

	Concentration of standard[a]	**Concentration in assay**
31.5 µl 57.2 µM DA + 268 µl GAD buffer[b]	6.0×10^{-6}	1.0×10^{-6}
30 µl 6.0×10^{-6} solution + 270 µl buffer	6.0×10^{-7}	1.0×10^{-7}
30 µl 6.0×10^{-7} solution + 270 µl buffer	6.0×10^{-8}	1.0×10^{-8}
150 µl 6.0×10^{-8} solution + 150 µl buffer	3.0×10^{-8}	5.0×10^{-9}
20 µl 6.0×10^{-8} solution + 180 µl buffer	6.0×10^{-9}	1.0×10^{-9}
20 µl 6.0×10^{-9} solution + 180 µl buffer	6.0×10^{-10}	1.0×10^{-10}
20 µl 6.0×10^{-10} solution + 180 µl buffer	6.0×10^{-11}	1.0×10^{-11}
Reference	Buffer only	0

a. Standards are diluted 1:6 in the assay.
b. See GAD treatment for buffer formulation.

3. Frog brain membrane preparation. Dilute the stock frog brain membrane preparation in ice-cold 50 mM TRIS, pH 7.4 to yield a final protein concentration of about 0.5 mg ml^{-1}.

Frog brain membrane preparation

1. Acquire 24 leopard frog (*Rana pipiens*) brains and place in beaker containing 30 ml water held on ice.

2. Place brains in glass homogenizer tube and homogenize using Teflon probe at 385 rpm. It is very *important* to keep the preparation *on ice* during the homogenization step.
3. Centrifuge homogenate at 54,000 × *g* for 15 min at 4°C.
4. Aspirate supernatant and re-suspend pellet in 30 ml ice-cold TRIS-citrate buffer (pH 7.1). Keep on ice.
5. Centrifuge homogenate at 54,000 × *g* for 15 min at 4°C.
6. Aspirate supernatant and re-suspend pellet in 20 ml per pellet (80 ml total volume) ice cold 50 mM Tris-HCl buffer (pH 7.1). Keep on ice.
7. Dispense membrane preparation into 2 ml aliquots and freeze immediately at -80°C; this preparation is stable for at least six months. Perform assay to determine protein concentration in order to establish appropriate dilution for receptor assay.
8. Prior to running assay, bring the required number of 2 ml aliquots of membrane preparation to room temperature and then incubate in 37°C water bath for 30 min (to dissociate any endogenous glutamate from receptors).
9. Centrifuge at 54,000 × *g* for 15 min at 4°C.
10. Aspirate supernatant and re-suspend pellet in 2 ml 50 mM Tris-HCl buffer (pH 7.1); the membrane preparation is now ready for use in the assay.

[N.B. 50 mM Tris buffer: dissolve 6 g TRIS in 1 l H_2O and adjust pH to 7.1 with citrate or HCl, depending on whether Tris-citrate or Tris-HCl is required.]

GAD treatment

All sample extracts must be pre-treated with glutamate decarboxylase (GAD) as follows:

1. Prepare GAD buffer (shelf life one week: 10 mM citrate, pH 5.0; 2 mM pyridoxal 5-phosphate; 200 mM NaCl.
2. Prepare GAD enzyme (Sigma-Aldrich, cat. #G3757) to a concentration of 100 units ml^{-1} in 10 mM citrate, pH 5.0; aliquot and store at -80°C.
3. Digest sample extracts by adding the following to dilution tubes: 50 µl sample extract, 40 µl GAD buffer, 10 µl GAD enzyme; then incubate on ice for 30 min. A positive control sample of 1 mM glutamate (in duplicate) must be run with each assay to confirm performance of the GAD enzyme digest.

[N.B.: It is important *when testing GAD-treated sample extracts (all extracts should be treated) that the standard curve as well as dilutions of the inter-assay calibration standard and samples be done using the GAD buffer instead of the TRIS buffer noted in the frog brain membrane preparation. Also, for those sample extracts subjected to SAX/SPE clean-up, the GAD treatment should follow the SAX clean-up.]*

Assay Procedure

For both conventional and microplate scintillation counting

1. Add the following to each of 96 wells *in the order given*:
   - 35 µl ice-cold 50 mM TRIS, pH 7.4 to pre-wet filters;
   - 35 µl [^{3}H]-kainic acid;
   - 35 µl DA standard or sample;
   - 105 µl membrane preparation.

[N.B: All standards and samples should be run in duplicate; arrange samples vertically in the plate to simplify organization after filters are punched into vials.]

2. Incubate for 1 hour at 4°C.
3. Filter the entire plate on top of the MultiScreen vacuum manifold.
4. Rinse each well once with 200 µl of ice-cold 50 mM TRIS, pH 7.4 buffer using multichannel pipette.

For both conventional and microplate scintillation counting
Details of counting and data analysis are as given for the STX receptor assay, except that final concentrations for unknown samples are determined by multiplying the calculated 'in assay' value by the assay dilution factor (i.e. 6.0), by 2 (i.e. GAD digest dilution factor), and then by any sample dilution factors.

12.4.3 Receptor binding assay for NSP (brevetoxins) and CFP (ciguatera) toxins

Note that there are close similarities between this assay and the procedure given above in the STX receptor binding assay (Section 12.4.1). Data analysis for the following methods can be performed with reference to the above procedure for STX binding.

Reagents
1. Standard binding medium (SBM) 50 mM HEPES (pH 7.4)
   - 130 mM choline chloride;
   - 5.5 mM glucose;
   - 0.8 mM magnesium sulphate;
   - 0.01% Emulphor EL-620 (non-ionic detergent as emulsifier);
   - 1 mM iodoacetimide;
   - 0.1 mM PMSF (phenylmethylsulfonyl fluoride);
   - 1 mM 1,10-phenanthroline;
   - 1 µM pepstatin A;
   - 1 mM EGTA;
   - make SBM with and without 1 mg ml^{-1} BSA.
2. Wash medium 163 mM choline chloride
   - 5 mM HEPES (to pH 7.4 with TRIS base);
   - 1.8 mM $CaCl_2$;
   - 0.8 mM $MgSO_4$;
   - 1 mg ml^{-1} BSA.
3. Brain-homogenization buffer (BHB) 0.32 M sucrose
   - 0.005 M Na_2HPO_4 (to pH 7.4 with phosphoric acid);
   - 1 mM iodoacetimide;
   - 0.1 mM PMSF;
   - 1 mM 1,10-phenanthroline;
   - 1 µM pepstatin A.

Membrane preparation
1. Homogenize two or three Sprague-Dawley rat brains at a time using 5 ml BHB per brain.
2. Centrifuge homogenate for 10 min at 3,000 × *g*. Save supernatant on ice and re-homogenize pellet.
3. Centrifuge re-homogenized pellet for 10 min at 3,000 × *g*.
4. Combine supernatant solutions and centrifuge for 60 min at 12,000 × *g*.

5. Discard supernatant and re-suspend pellet in a minimal amount of SBM without BSA.
6. Use microtitre protein assay to determine protein concentration of final solution.
7. Store membrane preparation in -80 ^{o}C freezer. At this temperature, membranes are stable for use in binding assays for months.

Assay Procedure

1. Add 0.4 ml toxin per sample to 1.5 ml microcentrifuge tubes. Use a serial dilution of standard for a 12 point calibration curve (10,000, 1,000, 500, 250, 100, 50, 25, 10, 5, 2.5, 1, 0 nM).
2. Add 0.5 ml of 2 nM [^{3}H]-PbTx3 to centrifuge tubes.
3. Add 0.4 ml SBM to centrifuge tubes. Also set up one triplicate sample with 10 µM unlabelled PbTx3 instead of SBM for determination of non-specific binding.
4. Add 0.1 ml of membrane preparation (at 0.5–1.0 mg ml^{-1} protein to give a final protein concentration of 50–100 µg ml^{-1}).
5. Close tubes and invert several times to mix, then incubate 1 h on ice.
6. Centrifuge at maximum speed in the micro-centrifuge.
7. Assay 0.1 ml of supernatant, carefully aspirate the remainder.
8. Rinse pellet with three drops of ice-cold wash medium, aspirate.
9. Clip bottom of tube containing the pellet into scintillation vial. Add scintillant, incubate overnight.
10. Vortex vigorously the next day and count in scintillation counter.
11. Analyse data.

12.5 Development and application of cytotoxicity assays

Cytotoxicity assays for several of the major phycotoxins are now well established in a few laboratories and they show promise as a rapid screening technique. In general, the quantities of cultured cells required for such bioassays can be readily scaled-up to deal with a sudden increase in demand, and the maintenance costs for continuing the cell line are modest. None of these assays has yet been validated by rigorous collaborative testing sufficient to warrant certification as an internationally recognized reference method, but several inter-laboratory comparisons have been carried out.

A tissue culture technique using an established mouse neuroblastoma cell line (Neuro-2A; ATCC, CCL131) has been developed for the assay of Na^{+} channel-blocking toxins (Kogure *et al*., 1988). The original assay was based on the microscopic examination of cell morphology and survival after incubation with reagents and putative Na^{+} channel blockers. This assay has been subsequently adapted and refined for rapid screening of these toxins by incorporating the use of a scanning spectrophotometer for colourimetric detection in microtitre plates (Gallacher and Birkbeck, 1992; Jellett *et al*., 1992, 1995). A toxicological evaluation using purified reference toxins (Jellett *et al*., 1995) has indicated that the cellular assay yields EC_{50} values for STX, NEO, GTX2 and epimerized mixtures of GTX2/GTX3 (Fig.12.8) that correlate quite well with their specific toxicity as determined by intraperitoneal mouse bioassays.

Two alkaloids, ouabain and veratridine, when added in combination to the neuroblastoma cell culture, increase the influx of Na^+ ions into the cells causing swelling and cell death. However, if a Na^+ channel-blocking agent is introduced, the antagonistic effect prevents Na^+ ion influx and the cells remain morphologically normal and in good growth condition. After staining with crystal violet, the intensity of the colour reaction is proportional to the quantity of Na^+ channel blocker present. An alternative assay based on the same principle, which was developed for the detection of Na^+ channel-blocking activity in bacterial culture supernatant, but could be readily reconfigured for detection of these toxins in algal or shellfish extracts, employs Neutral Red as the vital stain (Gallacher and Birkbeck, 1992). These functional assays target a highly specific subcellular unit (site-1, voltage-gated Na^+ channel), and are thus sensitive for channel-blocking agents that bind to this site (e.g. PSP toxins or TTX). However, if a shellfish extract contains both STX and PbTx, there will be a major under-reporting of both toxins using this procedure, as the Na^+ influx due to PbTx will be blocked by STX. Technical developments have extended this tissue culture assay to the site-5 Na^+ channel-activating toxins (PbTx and CTX) (Manger *et al*., 1993, 1995). The cytotoxicity assay detection limit for PbTx is 250 pg, and for purified CTX the sensitivity is in the low-picogram or even sub-picogram range. Sodium channel-activating toxins can be detected in 4–6 hours, but the

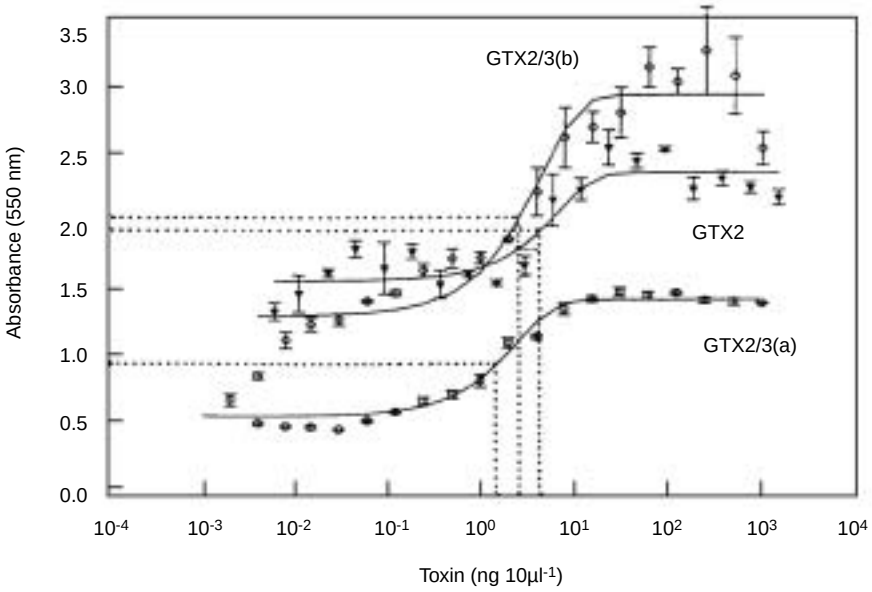

Figure 12.8
Dose-response profiles (absorbance of adherent stained cells ±SD versus concentration) of pure gonyautoxin-2 (GTX2) and two GTX2–GTX3 mixtures at different molar ratios: A, 1:2.5; B, 4:1. Each of the three profiles was obtained from a different bioassay. Dotted lines show the EC_{50} concentrations calculated for each.
Source: Jellett *et al.* (1995).

sensitivity can be enhanced with incubation for 22 hours (Garthwaite *et al*., 1996). These latter authors also showed the cell line NB41A3 (ATCC, CCL147) to be suitable for the assay. Cytotoxicity is assessed using a colourimetric detection system, whereby active cells reduce a tetrazolium compound (MTT) to a blue formazan product (Manger *et al*., 1995). Other cytotoxicity methods have used XTT (a water-soluble formazane reagent) for colourimetric determination of cell survival (Yasumoto *et al*., 1995).

Cell culture assays have also proven useful in screening for DSP toxicity in extracts of putatively toxic algae and shellfish. In the ciliate assay, live *Tetrahymena pyriformis* are exposed by adding partially purified DSP toxin extract to cultured cells and incubating for 24 hours (Shiraki *et al*., 1985). The concentration-dependent inhibitory effects on growth and acid phosphatase activity of the ciliate are used as an index of DSP toxicity.

In an alternative assay, freshly prepared rat hepatocytes are exposed to a lipophilic DSP toxin extract or to purified toxins, and the resultant damage to cell morphology (e.g. 'blebbing' and surface irregularities) are observed microscopically (Aune *et al*., 1991). The rate of release of lactate dehydrogenase can also be measured. Exposure to purified toxins yields distinctive results: OA and DTX1 cause severe blebbing and surface changes, whereas pectenotoxin-1 (PTX1) induces loss of microvilli and yessotoxin (YTX) yields formation of only tiny blebs.

A quantitative cytotoxicity bioassay using a human epidermoid carcinoma cell line (KB) was shown to be effective in detecting OA in mussel samples at a lower sensitivity limit of 50 µg OA kg^{-1} digestive-gland tissue in a 24-hour end-point assay (Tubaro *et al*., 1996*a*). This dose-dependent cytotoxicity assay is based on the metabolic conversion of a tetrazolium dye (MTT) to yield a blue-coloured formazan reaction product, which can be read for absorbance with a microplate scanning spectrophotometer.

Another recent modification to cell-based assays has led to the development of reporter gene assays for phycotoxin detection (Fairey *et al*., 1997). These assays employ cell lines such as Neuro-2A and pituitary GH4C1 (ATCC, CCL-82.2) that are stably transfected with a reporter gene construct comprised of the c-*fo*s regulatory region linked to the firefly luciferase gene. The c-*fos* early response gene is activated by changes in ion fluxes, such as those caused by certain phycotoxins. Induction of the reporter gene by a toxin yields a luciferase-catalysed light emission, which is read on a microplate luminometer. Selection of a cell line for detection of a given toxin is based on its expression of certain ion channel classes (i.e. Na^+, Ca^{++}). While this group of assays has yet to be evaluated for routine monitoring applications, it has proved useful in beginning to characterize the pharmacologic mode of action for bioactive fractions obtained from certain harmful algae (e.g. *Pfiesteria*) (Fairey *et al*., 1999).

12.5.1 Neuroblastoma assay for PSP toxins (saxitoxin analogues)

This functional cellular assay is based on the cytotoxicity of the Na^+ channel-blocking toxins, (e.g. PSP toxins and TTX). The procedure described in this section is employed in the MIST™ kit (Jellett Biotek, Dartmouth, Canada) for routine clinical analysis; other minor variations are used experimentally in other laboratories. Careful standardization of procedures and meticulous aseptic technique is required; therefore, the assay is best performed in a properly equipped tissue culture laboratory. Some clinical investigators using variants of the neuroblastoma assay

have stressed the importance of employing batch-standardized serum and titrating the alkaloids ouabaine and veratridine to achieve maximum reproducibility (Gallacher and Garthwaite, pers. comm.).

An AOAC inter-collaborative study of the MIST™ Quanti, the fully quantitative version of the MIST™ kit, was conducted internationally by eight independent laboratories (results submitted to the AOAC International Associate Referee for Seafood Toxins). The AOAC study demonstrated that the MIST™ Quanti system for the cell bioassay was as effective as the mouse bioassay in the detection and quantification of PSP toxins. Although distribution of these kits has been suspended due to obstacles encountered in maintaining cell stasis during long-distance shipping, the MIST™ PSP cytotoxicity testing system may be obtained as a complete technology transfer from Jellett Biotek (see Appendix 12.2).

The following is a detailed protocol for performing the neuroblastoma assay for PSP toxins (STX analogues). It is based on the MIST™ protocol, but applies equally to assays set up using the cell line Neuro-2A established in the laboratory.

Solutions

1. Tissue culture medium (CM):
   - RPMI 1640 with L-glutamine;
   - 10% foetal bovine serum (FBS);
   - 1% antibiotic solutions (10,000 units ml^{-1}penicillin G; 10,000 µg ml^{-1} streptomycin; 25 µg ml^{-1} amphotericin B may also be added if required to control infection by yeasts).
2. 0.25% trypsin in physiological saline stored frozen in small aliquots.
3. 10 mM ouabain octahydrate stored at room temperature (dark).
4. 1 mM veratridine in acidic (pH 2) distilled water stored frozen in small aliquots.

[N.B.: Solutions 3 and 4 must be filtered (0.2 µm) into sterile tubes immediately after preparation and before storage.]

5. Toxin decontamination solution: 1:1 bleach (sodium hypochorite solution) and 50% NaOH.
6. Phosphate buffered saline (PBS), pH 7.2 to 7.4, made fresh or autoclaved and stored at 5°C.
7. 10% formalin.
8. Gram's crystal violet.
9. 33% glacial acetic acid.

[Notes: Solutions 1 to 4 have limited shelf lives and should be made up only in small amounts as required. Components of CM are supplied sterile by manufacturers and must be maintained as such. Ouabain and Veratridine should be filter sterilized on preparation. Most of the solutions are highly toxic and should be handled with utmost care.]

Equipment

1. 37°C incubator.
2. microplate reader with filters to allow readings at 595 nm.
3. sterile 5 ml and 10 ml pipettes.
4. sterile 3–15 ml tubes for solutions.
5. single-channel pipettors (10–200 µl).

[N.B.: For inoculating the bioassay plates, a repeating multichannel micropipettor is highly recommended.]

6. sterile tips for the pipettors.
7. sterile tissue culture flasks and 96-well tissue culture dishes.
8. peel-and-stick Mylar sheets for 96-well dishes.
9. gloves, biohazard bags.

Cell culture

Cultures of Neuro-2A are grown in supplemented cell culture medium (CM) at 37°C in a humidified air-CO_2 atmosphere (5% CO_2). Cells are maintained in logarithmic growth phase in flasks, with passages established at a density of $1–5 \times 10^5$ cells ml^{-1} following trypsin dissociation. Passaging is required every two to four days.

Assay Procedure

[N.B.: As there are many hazardous or toxic components, it is advisable to wear gloves during the entire procedure. The cells are from a mouse cell line, but are classified as biohazardous because of their cancerous origin. Disposables that come into contact with these cells should be placed in a biohazard bag, then autoclaved or incinerated. Glassware should be autoclaved prior to washing. Any excess reagents, containers or tips that have come into contact with toxic components must be decontaminated by soaking for at least an hour in decontamination solution, followed by washing or by disposal in a biohazard bag.]

1. Dissociate the cells from the tissue culture flasks with 1–2 ml of trypsin solution. Re-suspend the cells in CM to a density of $1–5 \times 10^5$ cells ml^{-1} and inoculate 200 µl of cell suspension into each well of the 96-well plates.
2. Seal the plates with peel-and-stick Mylar sheets and incubate them at 37°C overnight.
3. The next day, prepare empty sterile 96-well plates for dilutions of standard and samples. Dilute pure STX with CM to a range of concentrations between 30 nM and 300 nM with a couple of concentrations beyond the active range on each end. This STX should be diluted just before use; it is not stable stored in the CM. In another plate, dilute the sample with CM to yield a range of 5 to 8 dilutions. Serial dilutions should be prepared based on the suspected toxicity (i.e. 1:2 dilutions if low toxicity is suspected to 1:10 dilutions for extracts with higher suspected toxicity).
4. Once the dilutions of the standard and the samples have been completed, remove the plate(s) containing the growing cells from the incubator and remove the Mylar sheet. It is best to keep a sterile lid on the plate of cells between additions, especially if working slowly. Add 10 µl of each dilution of either standard or sample to individual wells in the dish with the cells. Do not use any wells around the outer edge, and do not disturb the cells with the pipettor tips. Leave at least an additional two wells without standard or sample as controls, and mark inoculated wells on the dish and an associated master sheet.
5. Shake the plate gently, then add 10 µl of ouabain stock solution to each well to which either standard or sample dilutions have been added, and to the two or more controls.
6. Shake the plate gently, then add 10 µl of veratridine stock solution to each control well.
7. Shake the plate gently, then cover the plate(s) with a new sheet of Mylar and incubate for 8 hours to overnight at 37°C.

8. Remove the plate(s) from the incubator, and observe under an inverted microscope. Cells in the control wells (ouabain + veratridine only) should look swollen, granular and lysing, indicating that the plate is ready for development. Remove and discard the Mylar sheet.

[N.B: Both the decontamination solution and the formalin give off hazardous fumes, therefore the plate development should be carried out in a fume hood.]

9. Dump the contents of the plate(s) into the toxin decontamination solution and shake out the excess. Do not allow splashing of this solution into the plate as this will be instantly lethal to cells.
10. Wash away any lysed or non-adherent cells in PBS.
11. Immerse the plate(s) in 10% formalin and fix for 15 min.
12. Remove the plate(s) from the formalin and shake off the excess.
13. Flood the plate(s) with crystal violet and stain for 5 min.
14. Rinse out the stain using running tap water until no more excess stain is apparent in the rinse water. Shake off the excess water and allow the plate(s) to air dry or dry them in a gentle warming oven.
15. To reconstitute the plate(s), add 100 µl of 33% acetic acid to each well and wait for 1 hour.
16. Read the plate(s) spectophotometrically at 595 nm with a microplate reader.
17. To analyse the data, plot the absorbance for each concentration of STX standard and prepare a standard curve by fitting a line to the points using a four-parameter equation. Determine the concentration of STX that affects 50% of the cells (EC_{50}). This should not change from assay to assay (outside the variance limits ±20%), and will be independent of differences in the initial cell concentration. Find dilutions of each sample that are in the absorbance range of the dynamic part of the standard curve. Use the equation of the fitted standard curve to generate the concentration of STX equivalents in each dilution, then multiply by the inverse of the dilution factor to determine the concentration in the original sample. Average all dilutions that fall within the dynamic range of the standard curve to calculate the final result for each sample (and the standard deviation, if needed).

12.5.2 Neuroblastoma assay for NSP (brevetoxins) and CFP (ciguatera) toxins

The following procedures (Manger *et al*., 1993, 1995) are similar to those described in Section 12.5.1 for assaying the Na^+ channel-blocking toxins, including use of the same cell line and alkaloids, but the assay is configured to detect Na^+ channel activators (e.g. PbTx and CTX) by reducing the concentration of veratridine added, and thus the initial Na^+ influx. Detection is colourimetric, based on the ability of active cells to reduce the tetrazolium dye MTT to a blue-coloured formazan product.

Reagents

1. Mouse neuroblastoma cells: Neuro-2A (ATCC, CCL131).
2. Cell culture medium: RPMI 1640 complete medium (Sigma)
   - 10% heat inactivated foetal bovine serum (Gibco);
   - 2 mM glutamine (Sigma);
   - 1 mM sodium pyruvate (Sigma);
   - 50 µg ml^{-1} streptomycin (Sigma);

- 50 units ml^{-1} penicillin (Sigma).

3. Ouabain stock: 10 mM ouabain (Sigma) in H_2O, pH 2.
4. Veratridine: 1 mM veratridine (Sigma) in H_2O, pH 2.
5. Cell harvest solution (CHS): 0.5% trypsin/0.2% EDTA in PBS.
6. MTT (3–[4,5–dimethylthiazol-2-yl]–2,5–diphenyltetrazolium) stock: 5 mg ml^{-1} solution in PBS, pH 7.4; store at 4°C until use.
7. Stop reagent: DMSO (dimethylsulfoxide).
8. Brevetoxin (PbTx) standards: Dissolve in methanol for stock solution. Dilute 1:100 in cell culture medium prior to analysis. Make serial dilutions from this 1:100 dilute stock.
9. Ciguatoxin (CTX) standards or ciguatoxic fish extract (methanol fraction).

Cell culture

Cultures of Neuro-2A are grown in supplemented cell culture medium at 37°C in a humidified air-CO_2 atmosphere (5% CO_2). See also notes in Section 12.5.1.

Procedure

1. Harvest cells by exposing to CHS for 1–2 min, replace with medium, and gently wash cells from culture flask. Dilute and seed directly into a 96-well microtitre plate at a density of 1×10^5 in 200 µl growth medium, or perform an intermediate centrifugation ($2{,}000 \times g$) and re-suspension step to remove cell debris before seeding. Incubate for 16–24 hours.
2. Add 10 µl of each solution: sample/standard, ouabain stock and veratridine stock to each well. Test samples in triplicate at various dilutions. Process a minimum of five wells as ouabain/veratridine controls with no sample addition and five wells as untreated controls without sample, ouabain, or veratridine. Assess non-specific interactions and cytotoxicity with the use of sample without ouabain or veratridine. Add culture medium to make up for solutions not used in various controls. Incubate 4–22 hours at 37 °C for detection of Na^+ channel activators.
3. Remove overlaying medium from culture plates. *Do not wash* the plates.
4. Add 60 µl of a 1:6 dilution of MTT stock to each well. Incubate approximately 15 min at 37°C or until a dark formazan deposit is observed in control wells (can be as long as 30–45 min).
5. Remove overlaying medium from culture plates. *Do not wash* the plates.
6. Add 100 µl DMSO to each well.
7. Read absorbance *immediately* at 570 nm with reference at 630 nm.
8. Analyse data.

APPENDIX 12.1

Extraction methods and sample preparation

This section provides guidance on the extraction and preparation of phycotoxins for assay by *in vitro* biochemical or cytological methods. As most of these *in vitro* assays are validated and compared with alternative analytical techniques and mammalian bioassays by screening extracts prepared by standardized protocols, such as the AOAC (2000) method for PSP toxins, the detailed procedures may be obtained in Chapters 7–10 and 13. Only special notes and extraction procedures not referenced

elsewhere in this manual are given here. To avoid duplication, certain complementary information on the availability of reference materials and reagents may be found in Chapters 7–9. Many extraction procedures have been used to determine phycotoxins in shellfish and fish matrices, and also for toxin isolation. The choice of extraction procedure is critical for development and use of a sensitive robust assay, and incorporation of a procedure other than that recommended in the test kit or method literature will require extensive re-validation. Any new procedure must be checked for its ability to extract all chemical variants of a toxin group to be assayed, and the extraction efficiency must be established and documented. Each detection method may demand a specific sample extraction, or clean-up procedure, with care being taken to avoid the introduction of co-extracting compounds that may cause artefacts, especially with samples that exhibit 'non-standard' toxin profiles (e.g. extraction of samples containing both STXs and PbTxs for neuroblastoma assay). Garthwaite *et al*. (2001) have examined the potential of 70–90% aqueous alcohols to act as a universal solvent for extraction of phycotoxins for ELISA. This investigation showed the feasibility of this solvent system, but much work is needed to validate such a procedure.

In vitro assays for hydrophilic toxins

Preparation of shellfish tissues

The AOAC (2000) method for the extraction of hydrophilic toxins in boiling 0.1 M HCl and subsequent preparation of shellfish tissues for mouse bioassay is widely used and accepted as a standard method for regulatory purposes. For this reason, diagnostic assays for PSP toxins in shellfish are usually configured to be compatible with the use of extracts prepared according to this method (details provided in Chapter 13).

The MIST Alert™ for PSP toxins (Section 12.2.1.2) is designed for use with the AOAC (2000) extraction procedure, but an additional validated procedure was developed specifically for the application of this test to smaller samples directly in the field. According to recommendations provided by Jellett Biotek, the 'mini-AOAC' method is based on the extraction of a 10 g subsample of a representative 100 g homogenate of shellfish tissue. The method has been validated against the standard 100 g AOAC method with shellfish samples of several different toxin profiles (Jellett *et al*., 2001). This extraction protocol is also compatible with the use of the MIST Alert™ for ASP toxins, subject to the caveats discussed in Chapter 9 regarding the use of AOAC extracts in 0.1 M HCl for the instrumental analysis of DA.

As for the cytotoxicity assays and immunodiagnostic techniques, shellfish extracts prepared according to standard AOAC (2000) procedures can be used in the receptor binding assays (Sections 12.4.1 and 12.4.2) with no complex 'clean-up'. However, it is *important* that the sample pH is not <2, as this can result in a false-positive response. Furthermore, as much particulate material as possible should be removed either by centrifugation and/or filtration prior to performing filter manifold-based assays, otherwise the filter plate will clog rapidly.

For the assay of shellfish samples extracted in 50% aqueous methanol (see Chapter 9, Appendix 2), immunoassays may be performed following dilution of clarified extract to lower the sample methanol percentage to 10%. For analysis using the DA receptor binding assay, additional modifications are required. All shellfish extracts should be subjected to the SAX (strong anion exchange) solid phase extrac-

tion (SPE) clean-up protocol outlined by Hatfield *et al*. (1994), instead of the method given in Chapter 9, as the citrate elution solvent is not compatible with the receptor assay. Briefly, a 2 ml sample is loaded on to the SPE cartridge followed by washing with 5 ml of 0.1 M NaCl in 10% aqueous acetonitrile, the void volume flushed with 0.5 ml of 0.5 M NaCl in 10% acetonitrile, and the DA eluted with 5 ml of 0.5 M NaCl in 10% acetonitrile. All cleaned extracts *must* be treated with a GAD enzyme digest prior to performing the assay.

Preparation of plankton

The high sensitivity of *in vitro* methods permits the direct assay of soluble phycotoxins in seawater, and the supernatants of cell cultures. A simple 1:3 dilution of sample to reduce the salt concentration is often sufficient. Determination of intracellular toxin content, however, usually requires cell lysis. This is effectively achieved by either repeated freeze/thaw cycles, sonication, or prolonged vortex mixing/high-speed centrifugation.

The following plankton preparation method was originally developed for use with the SAXITOXIN TEST® immunoassay (Cembella and Lamoureux, 1993). The current protocol provides further details and modifications specifically suitable for use with the MIST Alert™ immunoassy for both PSP and ASP toxins (Silva *et al*., 2001, Cembella *et al*., 2001*a*, 2001*b*). Extracts of plankton can be prepared according to this method for testing with the STX receptor assay, and the technique is also applicable for the extraction of water-soluble toxins for analysis by chemical analytical methods (Chapters 7 and 9).

1. Collect phytoplankton using a plankton net, bucket, pump, Niskin bottle or vertically integrated plankton sampler. When using a plankton net, the mesh size should be as small as possible and no more the 20 µm. Regardless of collection method, it is usually necessary to further concentrate the sample. This is readily accomplished by seiving samples through a Nitex mesh mounted in a cylinder. If ASP-toxigenic pennate diatoms are being collected, cells must be concentrated on a smaller mesh (10 µm) or many will be lost by passing longitudinally through the seive. If only PSP-toxigenic phytoplankton are being sought, a 20 µm mesh size is adequate.
2. Fix subsamples with Lugol's iodine or 2% (final concentration) formalin-acetic acid solution and perform microscopic cell counts in duplicate using a Palmer-Maloney or Sedgewick-Rafter counting chamber. With natural phytoplankton assemblages (e.g. from net tows or pumped samples) determine the species composition and obtain cell counts of each putatively toxic species (e.g. *Alexandrium*, *Gymnodinium catenatum* and *Pyrodinium*) in the cell concentrate by microscopy. Calculate and record the volume of plankton suspension required to yield *c.* 1×10^5 (but not $<10^4$) cells of the potentially toxic species and harvest as a pellet.
3. Accumulate a moist pellet after rinsing cells collected on the surface of the Nitex mesh toward one corner side of the cylinder.
4. With a pipette, transfer the moist pellet to a 15 ml conical-bottom centrifuge tube and centrifuge at $5{,}000 \times g$ for 15 min at 4°C.
5. Gently aspirate away the supernatant so as not to disturb the pellet. The moist pellet can be immediately used to extract toxins or the sample can be preserved for later extraction by freezing quickly (on dry ice for 10 min, then storage at -20°C is recommended).

6. For toxin extraction, add 500 µl of 0.1 M acetic acid directly to the fresh or frozen-thawed pellet. Mix well with a pipette tip and transfer the suspension into a 1 ml microcentrifuge tube. This extract can be used immediately for testing by MIST Alert™ or it can be retained at 4°C for several days for later assay (see protocol in Section 12.2.1.1.2).

[N.B.: This crude extraction method without cell lysis appears to yield quantitative recovery of toxins from plankton cell suspensions for application to the MIST Alert™ but it has not been specifically validated for other assay or analytical methods, e.g. in Chapters 7 and 9. For alternative detection methods, the cells should be mechanically or ultrasonically disrupted as in the following steps.]

7. Sonicate cell suspension with an ultrasonicator at maximum intensity (10×10 s; >25 W) using a microtip probe. Immerse the bottom of the centrifuge tube in an ice/ethanol bath or use a cold block.
8. Confirm by microscopic examination that the cells are completely disrupted. If not repeat the sonication (step 7).
9. Centrifuge extract for 10 min at $10{,}000 \times g$ and collect the supernatant.
10. Filter supernatant through a 0.45 µm membrane (HAWP Millipore, or polycarbonate equivalent) mounted on a Luer-lock syringe, or centrifuge at $2{,}000 \times g$ through a 0.5 ml microcentrifuge cartridge (0.45 µm Ultrafree-MC Millipore, or equivalent).
11. To improve comparative quantitation for PSP toxin assay methods based on the calculation of 'toxicity equivalents' (e.g. STXeq), subsamples (200 µl) may be heated in sealed glass reaction vials in a heating block at 100°C for 10 min after addition of 1:10 v/v of 1 M HCl. This treatment effectively hydrolyses the low-potency N-sulfocarbamoyl toxins to carbamate derivatives.

Receptor binding assay for ASP toxins

Seawater-based extracts of plankton samples for DA determination can be prepared according to the protocol given in Chapter 9, Appendix 9.3, for the HPLC-FMOC method. There are, however, cases when it is desirable to subject plankton extracts to SAX/SPE clean-up, which favours application of aqueous methanol extracts. The following procedure for preparing 10% aqueous methanol extracts of plankton collected on glass-fibre filters provides enhanced versatility over seawater extracts while achieving comparable extraction efficiencies. Note that only the NaCl elution protocol of Hatfield *et al*. (1994) is compatible with the assay.

1. Tare a dry, conical glass grinding tube (Kimble/Kontes, Vineland, N.J., USA; cat. #885352-0023) on a balance, carefully place sample filter at bottom of tube, record weight (= wet filter weight).
2. Add desired amount of 10% aqueous methanol to grinding tube (2.5 ml for 25 mm diameter filter, 5 ml for 47 mm diameter filter), record weight, and mix tube to homogenize cell distribution.
3. Disrupt filter by grinding with fitted Teflon pestle at room temperature for 1 min at 250 rpm for 25 mm filters (2 min for 47 mm filters). Wash Teflon pestle with methanol and water between samples and dry with laboratory tissue.
4. Centrifuge for 1 min at about 45 x *g* to bring down filter particles and extraction solvent.
5. Rinse upper edges of tube with supernatant to bring down filtre particles adhering to glass.

6. Sonicate on ice slurry for 1 min for 25 mm filters (2 min for 47 mm filters) using a 500 W sonic disrupter with a microprobe at maximum setting. Rinse microprobe with methanol and water between samples and dry with laboratory tissue.
7. Transfer sonicated sample to 5 ml transport tube (USA Scientific Plastics, Ocala, Fla., USA, cat. #1505-1000) and centrifuge for 1 min at 8,000 × *g* to pellet filter particles.
8. Remove supernatant using 3 cc or 5 cc syringe and pass through 0.22 µm syringe filter into 2 ml or 5 ml cryovial.
9. Store samples at -20°C until analysis.
10. Use the following equations to calculate the total DA equivalents in an extract, which can then be used to calculate particulate DA concentrations:
    a) total extract vol. (ml) = [(wet filter wt (g) – dry filter wt (g))/1.03 (g ml^{-1})) + 10% methanol vol. (ml)];
    b) nmol DAeq in extract = [(total extract vol. (ml))(DAeq from assay (nM))]/1000; where dry filter wt = mean dry wt (n = 10) of the filter type being used; 1.03 g ml^{-1} = approx. density of seawater at 35 psu.

In vitro assays for lipophilic toxins

Preparation of shellfish tissues

Extraction of shellfish tissue samples for DSP toxin methods typically follows a procedure such as the extraction method developed by Lee *et al*. (1987) (see also alternatives in Chapters 8 and 13). This method involves extraction with 80% aqueous methanol followed by centrifugation at 3,000 x *g* for 15 min. The supernatant is stored at -20 ºC until needed. For immunoassays (Section 12.2.3), samples must be diluted to reduce the methanol concentration, but further clean-up is not required. Dilute extracts 1:1 with dH_2O for a final concentration of 40% aqueous methanol. Use 40% methanol in TRIS-buffered saline (TBS), or phosphate-buffered saline (PBS) for all subsequent sample dilutions.

For the protein phosphatase inhibition assay for DSP toxins and microcystin-LR (Section 12.3.1), homogenize shellfish tissues in 90% aqueous methanol in a 5 + 1 (v/w) solvent-to-tissue ratio. Homogenize for 1 min in a blender. Filter the suspension (Whatman 541 filter paper), and dilute for analysis (see Section 12.3.1). This extract can also be applied to the DSP ELISA following 1:1 dilution in PBS.

The American Public Health Association (APHA) extraction procedure for shellfish tissue was compared with an alternative protocol developed for extraction of a novel NSP toxin (Hannah *et al*., 1995). The APHA method (Irwin, 1970) uses a diethyl ether extraction, whereas the Hannah (1995) method involves an acetone extraction followed by a dichloromethane partition. Recovery (based on mouse bioassay toxicity) was consistently higher for the NSP toxin group using the acetone extraction than with the APHA method (see Chapter 13), and thus this method may also be preferred for *in vitro* assays.

Preparation of plankton

As for hydrophilic toxins, the high sensitivity of *in vitro* methods permits the direct assay of lipophilic toxins in seawater and the supernatants of cell cultures. Surprisingly large amounts of the lypophilic toxins may be detected in this supernatant fraction. Determination of intracellular toxin content requires cell lysis by repeated

freeze/thaw cycles, sonication, or prolonged vortex mixing/high-speed centrifugation. For the detection of lypophilic toxins, the addition of solvent to the system to help solubilize toxin is recommended; however, the choice of solvent is limited as issues of compatibility with *in vitro* methods may arise.

For immunoassay of plankton samples for DSP toxins (Section 12.2.3), extract concentrated biomass with 100% methanol at a 10 + 1 (v/w) solvent-to-tissue ratio. Sonicate sample (or mix vigorously and thoroughly on a Vortex mixer) and centrifuge for 15 min at 5,000 × *g*. For assay, dilute extracts 1:2.5 with TBS. Use 40% methanol prepared in TRIS-buffered saline (TBS) for all subsequent sample dilutions.

APPENDIX 12.2

Sources of test kits, standards and reagents

For PSP and ASP toxins

The RIDASCREEN^{R} immunodiagnostic test kit for PSP toxins is available from R-Biopharm, GmbH, Rösslerstrasse 94, D-1600, Darmstadt, Germany (www.r-biopharm.com).

The MIST^{TM} cytotoxicity assay and MIST Alert^{TM} immunodiagnostic tests for PSP and ASP toxins are available from Jellett Biotek 2002, Chester Basin, Nova Scotia, Canada; tel: +1 902-275-5760; e-mail: jjellett@ns.sympatico.ca.

Standard STX di-HCl calibration solution (100 µg ml^{-1}) can be obtained from the Division of Contaminants Chemistry, Natural Products and Instrumentation Branch (HFF-423), Food and Drug Administration, 200 C St, SW, Washington, D.C. 20204. USA.

For DSP toxins (okadaic acid and derivatives)

The DSP Check ELISA kit (SCETI Laboratories, Tokyo Japan) is also distributed under the RIDASCREEN® label, R-Biopharm GmbH, Rösslerstrasse 94, D-1600, Darmstadt, Germany (www.r-biopharm.com).

Reagents and supplies necessary for the protein phospatase assay for DSP toxins may be obtained from the following sources:

Alexis Biochemicals; tel: +1 800-900-0065; web: http://www.alexis-corp.com/

Costar, from VWR; tel: +1 800-932-5000; web: http://www.vwrsp.com/

IMB-NRC-Canada; tel: +1 902-426-8281; web: http://www.imb.nrc.ca/crmp/toxin-e.html

Rainin; tel: +1 800-472-4646; web: http://www.rainin.com/

Sigma; tel: +1 800-325-3010; web: http://www.sigma-aldrich.com/

United Lab Plastics; tel: +1 800-722-2499; web: http://www.unitedlabplastics.com/

Upstate Biotechnology; tel: +1 800-233-3991; web: http://www.upstatebiotech.com/.

Microcystins

Certain microcystins are available from Calbiochem, La Jolla, Calif., USA; tel: +1 800-854-3417; fax: +1 800-854-3417:

Microcystin-LR, cat. #475815-S

Microcystin-RR, cat. #475816-S

Microcystin-YR, cat. #475819-S
For further details consult Chapter 11.

CFP toxins (ciguatoxins)

Cigua-check™ solid phase immunobead assay is available via Hawaii Chemtect Int., Pasadena, Calif., USA (www.cigua.com). Ciguatoxin is not readily commercially available. The typical source of ciguatoxin is suspected ciguatoxic fish tissue. For further details consult Chapter 10.

NSP toxins (brevetoxins)

Certain NSP toxins may be purchased from the Calbiochem catalogue:
PbTx2, cat. #203732-S
PbTx3, cat. #203734-S
PbTx6, cat. #203737-S.

Some brevetoxin reagents, including research-grade competitive ELISA kits, toxin standards, antibodies, and a number reagents for NSP assay are available from Dr Dan Baden. Contact: HABLAB Reagents, University of North Carolina, Center for Marine Science, Harmful Algal Bloom Laboratories for Analytical Biotechnology, 5600 Marvin Moss Lane, Wilmington, N.C. 28409, USA; tel: +1 910-962-2300.

Antibodies against a number of the phycotoxins are also available from AgResearch Ruakura, East Street, Hamilton, New Zealand. Contact Dr Neale Towers; tel: +64 7-838-5187; fax: +64 7-838-5189. Several of the research assays are being reformulated into test kits in conjunction with Biosense Laboratories AS, Thormohlensgt 55, Bergen, Norway; tel +47 5554 3966; fax +47 5554 3771; web: http://www.biosense.com.

For phycotoxin standards and reference materials available from the Certified Reference Materials Program (CRMP), Institute for Marine Biosciences, National Research Council, Halifax, Canada, consult Appendices in Chapters 8 and 9.

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13

In vivo assays for phycotoxins

M. L. Fernández, D. J. A. Richard and A. D. Cembella

13.1 Introduction

The phycotoxins of principal concern to seafood safety involving human health and ecotoxicology in marine trophic webs include a wide diversity of compounds produced primarily by eukaryotic microalgae and cyanobacteria. These biologically active compounds range from components of low molecular weight, such as the secondary amino acid, domoic acid (mol. wt = 312), to complex polycyclic macromolecules, e.g. the cyclic polyether, maitotoxin (mol. wt = 3,422). Many phycotoxic syndromes result from simultaneous exposure to several closely related but structurally distinct toxin derivatives. Toxin potencies are also highly variable, not only between different classes of phycotoxins, but also among congeners of the same toxin family. Differences in toxin structure and potency, and complications introduced by complex organic matrices, clearly pose a challenge to the development and application of robust bioassays.

Whole-animal bioassays provide a measure of total toxicity based on the biological response of the animal to the toxins. Some oral feeding tests have been developed for phycotoxicity screening, and one such technique, the rat bioassay (Kat, 1983), is still used for DSP toxicity testing in a few European countries. Nevertheless, most mammalian bioassays for phycotoxins depend on intraperitoneal (i.p.) injection of an extract of shellfish in an aqueous solution, or in a less polar solvent followed by evaporation and re-suspension of the extract in a solvent compatible with injection. The choice of extraction solvent depends on the solubility properties of the toxins tested. Laboratory mice, which have been shown to react uniformly to toxins (e.g. Charles River CD-1 and CF-1, Swiss-Webster, etc.), are used routinely in phycotoxin bioassays. Following injection of the extract, subsequent observations are made to identify the time- and dose-dependent appearance of typical symptoms (morbidity and mortality) caused by the toxins. Toxicity values are generally interpolated from standard curves or tables, or from LD_{50} determinations. Survival time of mice is generally used for the measurement of overall toxicity, expressed in mouse units (MU) that can be converted into toxin-specific units [e.g. µg saxitoxin equivalents (STXeq)] based on the toxicity response when calibrated with toxin reference standards.

The principal advantage of a well-administered and properly calibrated mammalian bioassay, compared with physico-chemical analysis or many *in vitro* methods, is that the toxicity determination is directly proportional to human toxicity effects. Unfortunately, although this is true in theory, it is often difficult to accurately extrapolate results derived from an alternate route of toxin administration (often i.p.

injection) to human oral potency. Yet such bioassays can often be rapidly and conveniently conducted without complex equipment or the extensive clean-up procedures often required for more sophisticated analytical methods. In addition, toxicity determination does not require availability of standards for all analogues of toxicological interest, as is the case with instrumental analytical methods. Mammalian bioassays also screen inadvertently for the presence of unknown or poorly defined toxic components in the extract matrix, which may ultimately be found to have human health significance. For example, the first indications of the toxicity associated with the ASP syndrome, later related to domoic acid (Wright *et al*., 1989), were revealed in the course of routine AOAC mouse bioassays for PSP toxicity using acidic aqueous mussel extracts. Skilled bioassay analysts noted that the aberrant symptoms of ASP were distinguishable from the classic symptomology of PSP intoxication and consequent death.

Whole-animal bioassays have numerous inherent and operational deficiencies when applied to the accurate quantitation of phycotoxins. High capital investment and maintenance costs are often associated with the installation and operation of bioassay facilities involving live animals, especially mammals. Whole-animal bioassays are somewhat labour-intensive to perform and cannot be readily automated. An additional drawback of mammalian bioassays is the high variability among laboratories, due mainly to a number of variables that can affect the results, such as specific animal characteristics (strain, sex, age, weight), general state of health, diet, stress conditions, pH of the injected extracts and environmental conditions, such as temperature, humidity and photoperiod. Most of these parameters acquire special relevance when sample toxicity levels are near the regulatory limit. For these reasons, careful monitoring and optimization of the assay conditions is required to obtain reproducible and reliable assessment of toxicity and to reduce inconsistencies within and among laboratories. In the case of laboratories that are not equipped with the facilities needed to raise animals, they may be obtained from reliable and qualified suppliers. This requires careful planning so that animals of the proper size and condition are always available for assays. It should be noted that the use of laboratory animals is subject to regulations specifying the maintenance conditions (e.g. cage size, number of animals per cage, etc.), to ensure quality of the results and welfare of the animals (NIH, 1986).

Mammalian bioassays are susceptible to a host of artefacts and inaccuracies that can bias the validity of the results. False results (positive or negative) can occur due to interference by substances co-extracted during the sample preparation or to an inappropriate choice of extraction solvents or clean-up method. The co-occurrence of different toxins can yield synergistic or antagonistic biological responses, e.g. in the simultaneous presence of a Na^+ channel activator and a blocker. Many mammalian bioassays for phycotoxins have a poor dynamic range. Furthermore, dilution of the toxic analyte in the sample to achieve death times in the working range of the assay will also dilute other components in the matrix and this can lead to non-linearity of the standard dose-response curve. The assays tend to be more reliable for phycotoxins with small LD_{50} values and short death times (i.e. high acute toxicity). Compared with instrumental analytical methods, whole-animal bioassays are often less precise (±20% is typical under optimal conditions) and much less sensitive (by up to five orders of magnitude). For example, for the AOAC mouse bioassay for PSP toxicity, the acceptable regulatory limit for human consumption of shellfish in many countries (800 µg STXeq kg^{-1} soft tissue) is only twice the nominal toxicity

detection limit (*c*. 400 µg STXeq kg^{-1}). Despite these limitations, particularly with respect to PSP monitoring, historical evidence demonstrates that enforcement of the regulatory level and the use of the AOAC bioassay has provided a very strong level of confidence in the safety of shellfish. While the AOAC mouse bioassay does not provide qualitative information on toxin components, this is not a *prima facie* requirement for regulatory purposes. Nevertheless, despite the logistical and ethical problems inherent to mammalian bioassays, such assays are widely employed for phycotoxin monitoring in seafood since reliable and cost effective replacements are not yet available. For mammalian bioassays, only those that have been used in regulatory programmes are presented in detail in this chapter.

13.2 Paralytic shellfish poisoning (PSP)

Paralytic shellfish poisoning is a neurotoxic syndrome resulting primarily from the blockage of neuronal and muscular Na^+ channels. Binding to the Na^+ channel prevents propagation of the action potential that is essential to the conduction of nerve impulse and muscle contraction. In vertebrates, the peripheral nervous system is particularly affected; typical symptoms of poisoning include tingling and numbness of the extremities, then progressing to muscular incoordination, respiratory distress and muscular paralysis leading to death by asphyxiation in extreme cases. The PSP toxins include saxitoxin (STX) and approximately two dozen naturally occurring tetrahydropurine derivatives that vary in toxicity, chemical stability, and relative abundance in shellfish and dinoflagellates. This toxin group comprises: (a) highly potent carbamate toxins, including (STX), neosaxitoxin (NEO), gonyautoxins (GTX1–GTX4); (b) weakly toxic N-sulfocarbamoyl toxins (B1, B2, C1–C4); and (c) decarbamoyl (dc-) analogues of intermediate toxicity (see Chapter 7) (Fig. 13.1). Analysis of these compounds in natural matrices is complicated by the presence of multiple components that vary in specific toxicity (by up to two orders of magnitude), the susceptibility to chemical conversion during sample processing or storage, and the potential for substantial bioconversion that can occur in shellfish tissues. Nevertheless, these differences in specific toxicity among the various toxin derivatives do not necessarily have to be considered when determining integrated toxicity using assay methods that purport to yield an estimate of net toxicity.

13.2.1 AOAC mouse bioassay

The mouse bioassay for the determination of PSP toxicity was first applied by Sommer and Meyer (1937) to the assay of acidic extracts of mussels from California. In subsequent years, the general procedure was further standardized and validated in a series of inter-collaborative studies (McFarren, 1959). This reference method (AOAC, 1990) is the only procedure recognized internationally for quantifying PSP toxicity and it is used worldwide in PSP monitoring programmes, albeit with some variation in the acceptable regulatory limit for toxicity.

The PSP mouse bioassay for shellfish toxicity involves acidic aqueous extraction of the tissue (whole animal or selected organs) followed by i.p. injection of 1 ml of the extract supernatant into each of three mice. The mice are observed for classical PSP symptoms, such as jumping in the early stages, ataxia, ophthalmia, paralysis,

gasping, and death (usually in <15 min) by respiratory arrest. The time from initial injection to mouse death is recorded and the toxicity is determined (in MU) from Sommer's table. One mouse unit is defined as the amount of PSP toxin required to kill a 20 g mouse within 15 min. The bioassay is only quantitative when the mouse death occurs after 5 min. Samples yielding a mean death time of less than 5 min will require dilution so that death times of 5–7 min are achieved. For the inexperienced, several dilutions may be needed to obtain a result within this range. The precision of this assay is often given as ±20% (coefficient of variation) (Hurst *et al*., 1985), but precision of less than 10% is achievable with properly trained assayists. On dilution, accuracy will improve because of the reduction in Na^+ levels naturally present in tissue.

Following a standardization procedure, mouse units may be converted to toxicity units (µg STXeq kg^{-1}) soft tissue. The tolerance level agreed to by most countries is 800 µg STXeq kg^{-1} wet weight of edible tissue. Although the method has been extensively validated, there remain many caveats to be considered to minimize variability within and among laboratories. For this reason, bioassay standardization and periodic checking of the conversion factor (CF) is crucial. Interlaboratory comparisons are useful only if calibration with a known standard is carried out. The use of mouse units for comparison is ill advised because different strains of mice, environmental holding conditions, and assayist technique will affect the conversion values.

Another important parameter influencing the PSP toxin bioassay results is the pH during extraction. In the AOAC (1990) method, extraction in 0.1 M hydrochloric acid, followed by heating at 100°C, typically establishes a pH range between 2

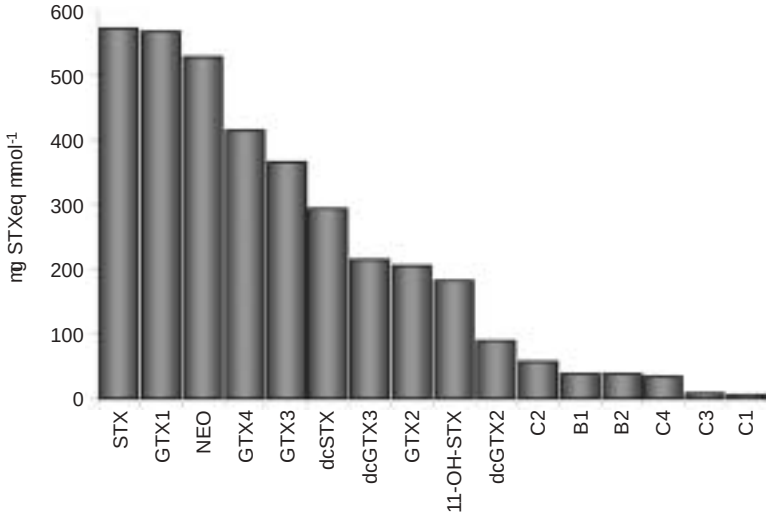

Figure 13.1
Specific toxicity of PSP toxin analogues [µg STXeq (saxitoxin equivalents) per micromole]. Values are calculated from corrected data (Oshima, pers. comm.) originally published in Oshima (1995), based on mouse bioassays performed with mouse strain ddY (sensitivity 1 MU = 0.23 µgSTXeq μmol^{-1}). STX, saxitoxin; NEO, neosaxitoxin; GTX, gonyautoxin; dc, decarbamoyl; 11–0H, 11-hydroxy; B–, C–, N–sulfocarbamoyl derivatives.

and 4. Under the hot acidic conditions required in the AOAC protocol, a substantial proportion of the labile but low-potency N-sulfocarbamoyl toxins (C1–C4, B1, B2) are converted to their respective high-toxicity carbamate analogues (approximately 40–60% conversion, depending on the tissue matrix buffering capacity: Cembella, unpublished data). Epimerization also occurs with this hot-acid treatment, resulting in the conversion of β- to α-epimers, but this usually has a minor effect on net toxicity. The PSP toxins are least stable at alkaline pH, yet heating under strongly acidic conditions (e.g. pH 2) can also lead to chemical transformation, with the degree of conversion depending on the pH (Nagashima *et al*., 1991). Between pH 3 and pH 4, PSP toxin components are in a range of optimal stability. Low pH of the injected extract can also lead to mouse bioassay artefacts caused by acidosis. A decrease in the acceptable pH range established in the AOAC (1990) procedure is highly advisable in order to reduce variability and improve reproducibility of the assay.

A crucial issue to resolve when applying the mouse bioassay or any other toxicity assay to the determination of PSP toxicity in various matrices is to establish *a priori* what is to be measured – maximum potential toxicity, actual *in situ* toxicity, or the toxicity of the prepared extract, such as that yielded by the AOAC protocol. In addition to the *in vivo* conversion of PSP toxins in shellfish to more potent analogues, particularly by transformation of the low-potency N-sulfocarbamoyl derivatives to carbamate analogues, such conversions may also occur during cooking or other processing of shellfish for human consumption. In fact, the standard AOAC procedure determines toxicity according to neither extreme, but instead yields an intermediate value. In view of the inconsistent and incomplete hydrolysis of N-sulfocarbamoyl toxin derivatives in PSP toxin-containing samples extracted according to the AOAC method, some authors have recommended a 10-fold increase (to 1.0 M) in the hydrochloric acid concentration (Hall *et al*., 1990). When followed by heating of the extract, this modification yields toxin values that closely approximate the maximum potential toxicity, but this approach has not been widely adopted because the existing AOAC (1990) procedure is strongly entrenched within regulatory structures. Furthermore, these elevated acid levels enhance the production of compounds which may be toxic to mice, thereby generating false-positive results. For human health protection, the use of relatively mild acid hydrolysis afforded by the AOAC protocol is usually adequate, because of the predominance of carbamate toxins in most samples, and the fact that there is a margin of security inherent in the regulatory limit, even when low-potency derivatives are dominant in the native toxin profile of the sample.

Many co-extracted substances can negatively affect biological assays, and the AOAC method is no exception. In early studies, Shantz *et al*. (1958) reported that high amounts of salts (mainly Na^+ ions) dissolved in the extracts can lower the apparent PSP toxicity in the mouse bioassay. Also, the addition of NaOH is sometimes necessary to raise the pH prior to injection, but this should be done carefully and drop-wise with stirring to avoid toxin decomposition caused by localized pH changes. The addition of excessive amounts of NaOH should be avoided.

The presence of high concentrations of certain metals is another potential source of interference with the PSP mouse bioassay. High levels of zinc (McCulloch *et al*., 1989; Cacho, 1993) can induce mouse deaths with apparent neurotoxic symptoms. The major difference between zinc toxicity artefacts and symptoms of classic PSP toxicity is that death usually occurs more than 15 min after injection of zinc-contaminated extracts. High levels of magnesium (neurotoxic symptoms) and

calcium (non-specific toxicant) can also cause mouse death when the AOAC method is used (Richard, unpublished data). Depending on the experience of the assayist, this effect could be falsely interpreted as low-level PSP activity.

The mouse bioassay unambiguously gives evidence of the toxic potential of a sample, because the application of higher toxin concentrations shortens the time to death of the laboratory animals. However, these biological tests reveal only the total PSP toxicity of a sample, expressed in MU (mouse units) kg^{-1} or STXeq kg^{-1} (Fig. 13.2).

13.2.1.1 *Mouse bioassay protocol*

The following procedure is adopted with only minor modifications from the AOAC (1990) mouse bioassay protocol for PSP toxicity. *Caution: Rubber gloves should be used when handling materials that may contain PSP toxins.*

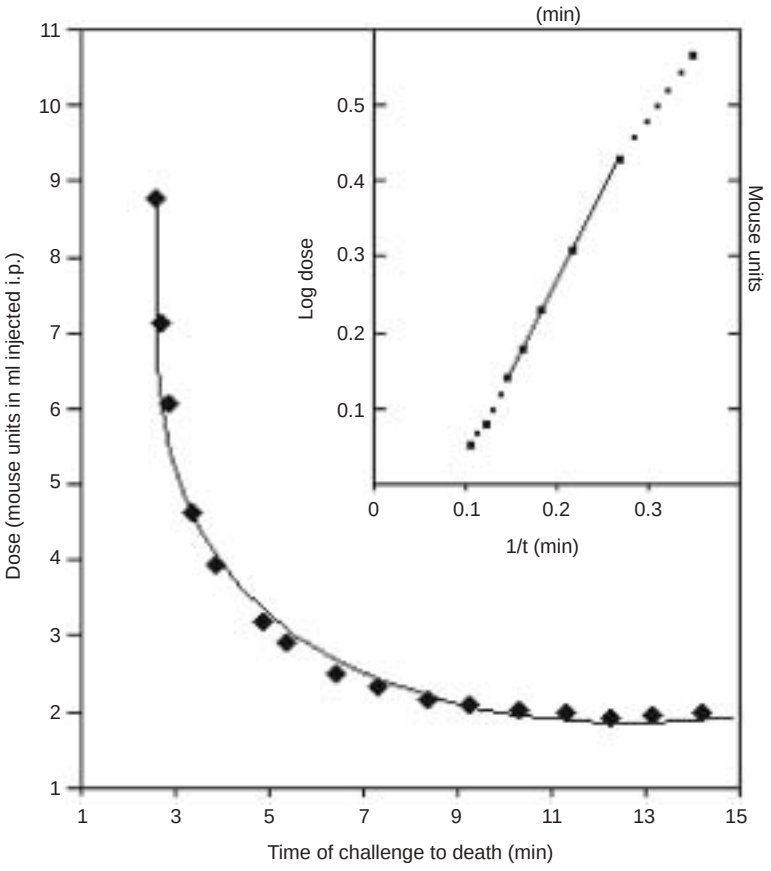

Figure 13.2
Relationship of the dose of PSP toxins to the time to death of mice.
Source: adapted from Luckas (2000).

Reagents and materials

(a) *Paralytic shellfish poison (STX) standard solution*: 100 µg ml^{-1} (US Food and Drug Administration), as acidified 20% alcohol solution. This standard is stable indefinitely when stored in a cool place.

(b) *Paralytic shellfish poison (STX) working standard solution*: 1 µg ml^{-1}. Dilute 1 ml of standard solution to 100 ml with H_2O. This solution is stable for several weeks at 3–4°C.

(c) *Mice*: use healthy mice (19–21 g) from a standard stock colony for routine assays. For mice <19 g or >21 g, apply the conversion factor (CF) to obtain the true death time (see Table 13.1). Do not use mice weighing more than 23 g and do not reuse assay subjects.

TABLE 13.1 Correction values for weight of mice

Mouse weight (g)	Mouse units (MU)	Mouse weight (g)	Mouse units (MU)
10	0.50	17	0.88
10.5	0.53	17.5	0.905
11	0.56	18	0.93
11.5	0.59	18.5	0.95
12	0.62	19	0.97
12.5	0.65	19.5	0.985
13	0.675	20	1.000
13.5	0.70	20.5	1.015
14	0.73	21	1.03
14.5	0.76	21.5	1.04
15	0.785	22	1.05
15.5	0.81	22.5	1.06
16	0.84	23	1.07
16.5	0.86		

Source: AOAC (1990)

Standardization of bioassay

1. Dilute 10 ml aliquots of 1 µg ml^{-1} standard STX solution with 10 ml, 15 ml, 20 ml, 25 ml and 30 ml H_2O, respectively, until i.p. injection of 1 ml doses into a few test mice yields a median death time of 5–7 min. The pH of the dilutions should be between 2 and 4 and must not be greater than 4.5. Test additional dilutions in 1 ml increments of H_2O, e.g. if 10 ml standard STX solution diluted with 25 ml H_2O kills mice in 5–7 min, test solutions dilutions as (10 + 24) ml and (10 + 26) ml as well.
2. Inject a group of 10 mice with each of two (or preferably three) dilutions which fall within the median death time of 5–7 min. Give a 1 ml dose to each mouse by i.p. injection and determine death time as the time elapsed from completion of injection to the last gasping breath of the mouse. Repeat the assay one or two days later, using dilutions prepared above which differed by 1 ml increments of H_2O. Then repeat the entire test, starting by testing dilutions prepared from fresh working standard solution.

3. Calculate median death time for each group of 10 mice injected with each dilution. If all groups of 10 mice injected with any one dilution gave median death times <5 min or >7 min, disregard results from this dilution in subsequent calculations. However, if any groups of 10 mice injected with a given dilution gave median death times between 5 min and 7 min include all groups of 10 mice assayed at this dilution, even though some of the median death times may be <5 min or >7 min. From the median death time for each group of 10 mice at each selected dilution, determine the MU ml^{-1} from Sommer's table (see Table 13.2). Divide calculated µg PSP toxin (STX) ml^{-1} by MU ml^{-1} to obtain the CF, expressed as µg PSP toxin (or µgSTXeq) per MU. Calculate the average CF value, and use this as a reference to verify routine assays. Individual CF values may vary significantly even within a given laboratory if the assay techniques and the condition of the mice are not rigidly controlled.

Verification of PSP toxin conversion factor for routine assays

Check CF value periodically as follows: if shellfish tissues are assayed less than once a week, determine the CF value on each day assays are performed by injecting five mice with the appropriate dilution of working standard. If assays are carried out on several days during a given week, only one verification need be made per week using a dilution of standard such that the median death time falls within 5–7 min. The CF thus determined should agree with the average value within ±20%. If the CF value does not lie within this range, complete the group of 10 mice by adding five additional specimens to the five already injected, and inject a second group of 10 mice with the same dilution of standard. Average the CF determined for the second group with that of the first group – this is the new CF value.

Repeated checks of CF value normally produce results within ±20%. If wider variations are found frequently, the possibility of uncontrolled or unrecognized variables in the technique or in the response of mice to PSP toxin should be investigated before proceeding with routine assays.

Sample preparation and extraction

1. *Clams, oysters, and mussels, etc. (whole tissues)*: Thoroughly clean the outside of shellfish with freshwater. Open by cutting adductor muscles. Rinse inside with freshwater to remove sand or other foreign material. Remove meat from the shell by separating adductor muscles and tissue connections at the hinge. Do not use heat or anaesthetics before opening the shell, and do not cut or damage the soft tissues at this stage. Collect 100–150 g of shellfish tissue in a glazed dish. As soon as possible, transfer tissues to a No. 10 sieve without layering, and drain for 5 min. Pick out pieces of shell and discard drainings. Grind tissues in household-type grinder with 1/8 inch-1/4 inch (3–6 mm) holes or in a blender until homogeneous.
2. *Scallops, etc. (selected tissues)*: Separate edible tissues (e.g. adductor muscle) and apply the extraction procedure to this portion alone. Drain and homogenize as in step 1.
3. *Canned shellfish*: Place entire contents of can (tissue and liquid) in a blender and blend until homogenous or grind three times through a meat chopper. For large cans, drain tissues for 2 min on a No. 8–12 sieve and collect all liquid. Determine wet weight of tissue and volume of liquid. Recombine portions of each sample in proportionate amounts and blend (or grind) until homogeneous.

TABLE 13.2 Sommer's table (959.08) relating mouse death times to toxicity units

Death time (min:s)	Mouse units (MU)	Death time (min:s)	Mouse units (MU)
1:00	100	5:00	1.92
1:10	66.2	5:05	1.89
1:15	38.3	5:10	1.86
1:20	26.4	5:15	1.83
1:25	20.7	5:20	1.80
1:30	16.5	5:30	1.74
1:35	13.9	5:40	1.69
1:40	11.9	5:45	1.67
1:45	10.4	5:50	1.64
1:50	9.33	6:00	1.60
1:55	8.42	6:15	1.54
2:00	7.67	6:30	1.48
2:05	7.04	6:45	1.43
2:10	6.52	7:00	1.39
2:15	6.06	7:15	1.35
2:20	5.66	7:30	1.31
2:25	5.32	7:45	1.28
2:30	5.00	8:00	1.25
2:35	4.73	8:15	1.22
2:40	4.48	8:30	1.20
2:45	4.26	8:45	1.18
2:50	4.06	9:00	1.16
2:55	3.88	9:30	1.13
3:00	3.70	10:00	1.11
3:05	3.57	10:30	1.09
3:10	3.43	11:00	1.075
3:15	3.31	11:30	1.06
3:20	3.19	12:00	1.05
3:25	3.08	13:00	1.03
3:30	2.98	14:00	1.015
3:35	2.88	15:00	1.000
3:40	2.79	16:00	0.99
3:45	2.71	17:00	0.98
3:50	2.63	18:00	0.972
3:55	2.56	19:00	0.965
4:00	2.50	20:00	0.96
4:05	2.44	21:00	0.954
4:10	2.38	22:00	0.948
4:15	2.32	23:00	0.942
4:20	2.26	24:00	0.937
4:25	2.21	25:00	0.934
4:30	2.16	30:00	0.917
4:35	2.12	40:00	0.898
4:40	2.08	60:00	0.875
4:45	2.04		
4:50	2.00		
4:55	1.96		

Source: AOAC (1990)

Weigh 100 g of well-mixed sample into a tared beaker. Add 100 ml 0.1N HCl, stir thoroughly and check pH (should be <4.0, preferably *c*. 3.0). If necessary, adjust pH as indicated in this section. Heat mixture and boil gently for 5 min, then cool to room temperature. Adjust cooled mixture to pH 2.0–4.0 (never >4.5), as determined by BHD Universal Indicator, phenol blue, Congo red paper, or by pH meter. To lower pH, add 5N HCl drop-wise with stirring; to raise pH, add 0.1N NaOH drop-wise with constant stirring to prevent local alkalinization and consequent destruction or conversion of PSP toxins. Transfer mixture to a graduated cylinder and dilute to 200 ml. Return mixture to the beaker, stir to homogeneity, and allow to settle until a portion of the supernatant is translucent and can be decanted free of solid particles large enough to block a 26-gauge hypodermic needle. If necessary, centrifuge the mixture or supernatant for 5 min at 3,000 × g or filter through paper to obtain enough liquid to perform the bioassay.

Mouse assay

Intraperitoneally inject each test mouse with 1 ml of acidic extract. Note the time of inoculation and observe mice carefully for the time of death as indicated by the last gasping breath. Record death time by stopwatch or clock with sweep second hand. One mouse may be used for the initial determination, but two or three are preferred. If death time or median death time of several mice is less than 5 min, prepare dilutions to obtain death times of 5–7 min. If death time of one or two mice injected with the undiluted extract is greater than 7 min, a total of three mice must be inoculated to establish sample toxicity. If large dilutions are necessary, adjust the pH of the dilution by drop-wise addition of dilute HCl (0.1 N or 0.01 N) to pH 2.0 to 4.0 (never >4.5). Inoculate three mice with a dilution that gives death times of 5–7 min.

Calculation of toxicity

Determine median death times of mice, including survivors, and from Sommer's table (Table 13.2) determine the corresponding toxicity in mouse units (MU). If test animals weigh <19 g or >21 g, apply a correction for each mouse by multiplying MU corresponding to death time by the weight correction factor; then determine median number of MU for this group. (*Consider death time of survivors as >6 min or equivalent to <0.875 MU in calculating the median.*) Convert MU to µg PSP toxin (STXeq) by multiplying by the CF value:

$$\mu g\ STXeq\ kg^{-1}\ tissue = (\mu g\ ml^{-1}) \times dilution\ factor \times 2{,}000$$

Consider any value greater than 800 µg STXeq kg^{-1} as hazardous and unsafe for human consumption.

13.2.2 Fly bioassay

The house fly (*Musca domestica*) bioassay (Ross *et al*., 1985) was developed as a viable alternative to the AOAC mouse assay for screening PSP toxins in shellfish. This method counters many of the disadvantages of the mouse bioassay, including those related to the high cost of mammalian bioassays, the difficulties in transporting and maintaining mice in acceptable facilities, animal rights concerns and the interference due to salt effects. In brief, flies are temporarily immobilized on a cold plate and injected (in batches of 20) with standard AOAC extracts in a dilution series. After 10 min, flies are binary scored for motor ability -0: free movement and/or

ability to flip from dorsal position; 1: positive, lack of motor ability. Interpretation of the results is based on the fact that there is a linear relationship between the probit of the fraction of flies affected and the logarithm of the dose. The method has proved to be reliable, with a mean ED_{50} of 0.25 ± 0.02 µg ml^{-1} (381 pg per fly) when tested with USFDA-certified STX standard. A collaborative study involving the assay of clam samples contaminated by PSP toxins and extracted according to the AOAC protocol was undertaken to compare the fly assay, the AOAC mouse assay, and a fluorescence-detection HPLC method. The fly bioassay showed a high degree of congruence with the mouse bioassay results, often with slightly higher values, but with a lower detection limit (250 µg STXeq kg^{-1}) for shellfish extracts than with the mouse bioassay (approximately 350–400 µg STXeq kg^{-1}). Unfortunately, in spite of the fact that attempts were made to introduce the use of the fly bioassay, particularly in developing countries in Asia, this method never achieved common usage. Perhaps one of the difficulties is the high degree of manual dexterity required for the injection of minute quantities (1.5 µl) of sample into the abdomen-thorax junction without damage to the test subject.

13.3 Amnesic shellfish poisoning (ASP)

Amnesic shellfish poisoning (ASP), caused by the neurotoxin domoic acid, was first identified in Canada in shellfish extracts from eastern Prince Edward Island (Wright *et al*., 1989). The phycotoxic syndrome known as amnesic shellfish poisoning was named for the memory loss associated with extreme cases of human intoxication from shellfish contaminated by domoic acid. Domoic acid is an excitatory amino acid derivative acting as a glutamate agonist on the kainate receptors of the central nervous system. This secondary amino acid is considered to be a more potent neuroexcitor than kainic acid, which when systemically injected into specific parts of the brain is known to have degenerative effects. Domoic acid is considered to be the primary toxin involved in ASP, although isomeric forms (e.g. iso-domoic acid) of lesser potency also occur naturally.

13.3.1 Bioassay protocols

The AOAC mouse bioassay for PSP toxicity can also detect domoic acid at concentrations *c*. 40 µg ml^{-1} injected. This procedure was initially used when ASP toxicity was first detected in leftover mussels implicated in human poisonings, and to direct the purification scheme in identifying domoic acid as the cause of ASP in shellfish extracts from eastern Prince Edward Island, Canada (Wright *et al*., 1989). The AOAC extraction procedure can yield substantial recovery of domoic acid from shellfish tissue (*c*. 80%), and subsequent modification of the extraction method using methanol:water improves extraction efficiency, but the tolerance level established in Canada and subsequently adopted by most other countries is 20 µg g^{-1}. Therefore the AOAC bioassay procedure is too insensitive to be used with confidence for regulatory purposes to quantify this toxin and it has been abandoned in favour of chemical analytical methods (see Chapter 8) by most regulatory authorities.

Under certain circumstances, a rat or mouse bioassay could be used to detect the presence of low-level domoic acid by careful monitoring of selected symptoms (Tasker *et al*., 1990; Doucette *et al*., 2000), but such a method would not meet

established criteria for regulatory purposes. Where all other means of analysis are unavailable, special extraction techniques designed to increase sensitivity (e.g. extraction of the digestive gland only) can be used in combination with the mouse bioassay and observing for symptoms as described in this chapter. This method can only be described as qualitative at best and should not be used instead of the universally accepted chemical analytical methods.

The typical signs of the presence of domoic acid in the AOAC bioassay procedure are a unique scratching of the shoulders by the hind leg syndrome, followed by convulsions. Clinical signs of domoic acid toxicity in mice, in the following general chronological order, may include circling, head nodding, mastication, forelimb tremor, 'wet dog' shakes, loss of postural control, profuse salivation, 'praying' and rearing, scratching of the scapular region by the ipsilateral hind paw, myoclonic jerks, barrel rotation, generalized tremor, kyphosis, rigidity, general clonic-toxic convulsions, and death. The time of observation, as opposed to that of the AOAC procedure for PSP toxicity, should be extended to 4 hours. In the original 1987 incident, mouse deaths associated with mussels containing domoic acid were never observed after 135 min (Quilliam *et al*., 1989; Todd, 1990).

13.4 Diarrhetic shellfish poisoning (DSP) and related polyether toxin syndromes

Three groups of polyether compounds have been historically included in the 'DSP toxin complex': (a) okadaic acid (OA) and analogues such as the dinophysistoxins, DTX1, DTX2 and acyl-derivatives (DTX3); (b) pectenotoxins (PTXs) — separable into two groups, one having a cyclic ester structure ('macrolide') and the other a 'seco acid' group, in which the ester bond in the cyclic structure is hydrolysed; and (c) yessotoxins (YTXs), disulfated polyether compounds (see Chapter 8). These compounds have a relatively high molecular weight and share lipophilicity and solubility properties, being extracted with the same generic procedures. The traditional DSP mouse bioassay involving acetone extraction (Yasumoto *et al*., 1978) can detect not only OA and DTXs, but also PTXs and YTXs that may co-occur with OA in phytoplankton and shellfish. For this reason, PTX and YTX have been often associated with 'DSP'. However, their biological activity is different from that of the OA group, and only toxins belonging to the latter group have been reported to produce diarrhoeic effects in humans (i.e. classic DSP symptoms). There is general agreement in the classification of OAs and DTXs as DSP toxins, but the inclusion of PTXs and YTXs in the 'DSP complex' has always been a subject of great controversy. The toxicity values for various lipophilic phycotoxins found in shellfish and toxigenic microalgae are presented in Table 13.3.

In humans, typical symptoms following the consumption of DSP toxin-contaminated shellfish include acute diarrhoea, nausea, vomiting and, in some cases, abdominal pain. Although no human mortalities from DSP have been reported, the affliction can be highly debilitating for several days. Okadaic acid and certain DTXs are potent inhibitors of at least two sub-classes of protein phosphatases (PP1 and PP2A) and this mode of action may be linked to the observed diarrhoea, degenerative changes in absorptive epithelium of the small intestine, and to tumor promotion (Fujiki *et al*., 1988). Toxicological and epidemiological data on PTX and YTX are scarce, and the action mechanism of these toxins has not yet been fully elucidated.

TABLE 13.3 Acute toxicity (LD_{50}) of various lipophilic phycotoxins after i.p. injection into mice. Only those lipophilic toxins found in shellfish and the corresponding toxigenic microalgae are included; toxicity values for ciguatoxins and other analogues found primarily in fish tissues are available from Lewis (1995)

Toxin group	Analogue	Toxicity (μg kg^{-1})	Pathology
Okadaic acid	OA	200[a]	Diarrhoea; tumour promotion
Dinophysistoxin	DTX1	160[a]	Diarrhoea
	DTX3	500[a]	Diarrhoea
Pectenotoxin	PTX1	250[a]	Hepatotoxic
	PTX2	230[a]	Hepatotoxic; diarrhoea
	PTX3	350[a]	*
	PTX4	770[a]	*
	PTX6	500[i]	*
	PTX7	>5 000[j]	*
	PTX8	>5 000[j]	*
	PTX9	>5 000[j]	*
Yessotoxin	YTX	100[a]	Cardiotoxic
	HydroxyYTX	500[b]	*
	TrinorYTX	220[b]	*
	HomoYTX	100[c]	*
	45-hydroxyhomoYTX	500[c]	*
	DesulfoYTX	500[d]	*
	CarboxyYTX	500[e]	*
Azaspiracid	AZA	200[f]	Diarrhoea
	AZA2	110[g]	Diarrhoea
	AZA3	140[g]	Diarrhoea
	AZA4	470[h]	Diarrhoea
	AZA5	1 000[h]	Diarrhoea
Gymnodimine		96[k]	Unconfirmed
Brevetoxin	BTX-B1	50[i]	Neurological
	BTX-B2	300[i]	Neurological
	BTX-B3	>300[i]	Neurological
Spirolide	B	200[l]	Unconfirmed
	des-methyl–C	40[m]	Unconfirmed

* In the case of PTX and YTX groups, oral toxicity and cytotoxicity studies have been carried out only for PTX1, PTX2 and YTX. For other analogues, the pathology is likely to be similar or identical to that of the parent compounds, but is currently undefined.

Sources: a, Yasumoto *et al*. (1989); b, Satake *et al*. (1996); c, Satake *et al*. (1997); d, Daiguji *et al*. (1998); e, Ciminiello *et al*. (2000); f, Satake *et al*. (1998); g, Ofuji *et al*. (1999*a*, 1999*b*); h, Ofuji *et al*. (2001); i, cited in Yasumoto (2000); j, Sasaki *et al*. (1998); k, Miles *et al*. (1999); l, Hu *et al*. (1995); m, Richard, Cembella and Quilliam, unpublished data.

By i.p. injection into mice, PTX1 has been shown to cause damage to the liver but no diarrhoea was observed (Terao *et al*., 1986). Pectenotoxin-2 is known to elicit extremely potent cytotoxic activities (at nanomolar to picomolar concentrations) against numerous human cancer-cell lines (Jung *et al*., 1995) and by oral administration into mice, can cause diarrhoea and severe injuries to the liver and intestine, with oral toxicity very similar to i.p. toxicity (230 µg kg^{-1}) (Ishige *et al*., 1988). As members of both the OA and PTX groups are produced by *Dinophysis* species, it is very difficult to assess separately the contribution of PTX to human intoxication. Recently, the presence of PTX2 seco-acid in shellfish was correlated with a toxic outbreak in Australia (Quilliam *et al*., 2000), but the co-occurrence of DTX esters makes this relationship to illness difficult to confirm.

With respect to YTXs, the i.p lethal dose ranges from 100–500 µg kg^{-1}, depending on the analogue, with the heart being the target organ (Terao *et al*., 1990). Oral toxicity is at least an order of magnitude lower than i.p. toxicity (Ojino *et al*., 1997, Aune *et al*., 2001). Diarrhoea has not been observed by oral administration, therefore the appropriateness of classifying these compounds as 'DSP toxins' is questionable. Studies carried out with human lymphocytes suggest that YTX acts on cyclic adenosine monophosphate, increasing the cytosolic levels of calcium (De la Rosa *et al*., 2001).

Although the precautionary principle suggests that the presence of the three groups of toxins should be monitored in shellfish, there is no general agreement concerning which toxins should be included in the regulatory programmes (see Chapter 24). Furthermore, the definition of acceptable testing procedures, the standardization of methods and action limits to be applied for regulatory purposes represents a difficult administrative and scientific challenge. The current situation is far from ideal, and leads to inconsistencies and a lack of equivalence between the toxin control approaches used by different countries. An important advance in the European Union was the approval of Decision 2002/225/EC establishing the maximum levels and quantitative methods for OA and DTX toxins, pectenotoxins, yessotoxins and azaspiracidsin bivalve molluscs, echinoderms, tunicates and marine gastropods (see Chapter 24).

13.4.1 Bioassay protocols

Despite the worldwide application of mammalian bioassays for DSP toxicity, there are large differences in the performance of the procedures and in the classification criteria employed among countries. Assay selectivity, specificity and toxin recovery depends greatly on the selection, purity and ratios of the organic solvents used for extraction and clean-up steps (see also Chapter 8). Bioassay procedures as diverse as the oral dosage rat bioassay and the i.p. injection mouse assay are not strictly homologous, because the former technique quantifies only the diarrhoeagenic effect of certain toxin components (OA and analogues), whereas the latter assay yields an estimate of overall 'DSP toxicity'. Most extraction procedures applied to DSP toxins in shellfish samples can also co-extract neurotoxic shellfish poisoning (NSP) toxins and certain other lipophilic ichthyotoxins that can yield non-specific artefacts in mammalian bioassays. There may also be interference by free fatty acids, which can vary with the particular shellfish species, as well as spatially and temporally. These components may be toxic to mice by i.p. injection, yielding false-positive reactions

for 'DSP toxicity', but innocuous orally to humans and negative by the rat oral bioassay (Takagi *et al*., 1984; Lawrence *et al*., 1994).

The mouse bioassay for DSP toxicity is considered to be quantitative, although as the prolonged observation time would suggest, it is less reliable as a determinant of acute toxicity than is the corresponding AOAC mouse assay for PSP toxicity. Furthermore, the precise cause of death in rodents by i.p. administration of DSP toxins is not yet known. Phosphatase inhibition activity is strongly indicated as the toxicity mechanism leading to death, but the results of the i.p. bioassay cannot be simply extrapolated to quantify human oral toxicity or diarrhoeic symptoms.

The DSP toxicity procedure as originally established (Yasumoto *et al*., 1978) involved the calculation of i.p. toxicity in MU, with 50 MU kg^{-1} of whole soft tissue accepted as the regulatory limit in Japan. A mouse unit was defined as the minimum quantity of toxins required to kill two of three mice within 24 hours of i.p. injection; this corresponds to 3.2 µg DTX1 or 4 µg OA for the ddY mouse strain used in Japan, but may vary somewhat with the strain. As the calculation of toxicity using LD_{50} methods can be laborious and time-consuming, regulatory laboratories in most countries currently use mouse survival time for the evaluation of DSP toxicity. There is no international consensus on the appropriate observation period – the acceptable criteria can vary from 'two of three mouse deaths in less than 5 hours' to 'two of three mouse deaths in less than 24 hours'. Such decisions are both operational and administrative, but they are also somewhat dependent on the specific procedure for sample preparation prior to mouse bioassay, as various components of differing toxicity may be selectively extracted.

On the basis of an interlaboratory study of the mouse bioassay (adult mice) and the suckling mouse test (Marcaillou-Le Baut *et al*., 1985), the survival time considered as a positive result (adult mouse assay) was reduced from 24 hours to 5 hours. The study showed that the survival time of adult mice beyond 5 hours corresponded to a toxicity lower than that revealed by the suckling mouse test, and that the survival of three of three mice for more than 5 hours was considered a negative response. In addition, the same investigators studied the relation between the survival time of three mice and OA concentration for observations of death time more than 5 hours, and found that less than 4 µg OA in an extract matrix was necessary to yield a death time greater than 5 hours. Consequently, a mouse survival time of more than 5 hours was considered lower than the regulatory action level, and therefore a negative result for DSP toxicity (Marcaillou-Le Baut and Masselin, 1990).

Further studies comparing mouse bioassay results and HPLC-FD determination of OA showed that survival times of around 5 hours corresponded with an OA concentration of approximately (and in some cases above) 2 mg OA kg^{-1} of hepatopancreas. Vieytes *et al*. (1996) showed a relation between the death times in the mouse bioassay and values obtained in HPLC analysis. An analysis of 127 samples by both methods showed that reduction of the observation time from 24 hours to 5 hours could increase (by 35–76%) negative bioassays for toxin levels between 2 mg OA kg^{-1} and 3 mg OA kg^{-1} of hepatopancreas. Vale and Sampayo (1996) found that, although death times between 5 hours and 24 hours usually corresponded to OA + DTX2 concentrations of about 2 mg OAeq kg^{-1} of hepatopancreas, in some samples, appreciable amounts of DSP toxins (>2 mg OAeq kg^{-1} of hepatopancreas) resulted in survival times longer than 5 hours.

When evaluating the level of consumer protection provided by the different toxicity criteria, the considerable degree of uncertainty associated to the mouse bio-

assay protocols should be taken into account. Although the purpose of the assay is to assess toxicity of the edible part of the molluscs (usually the whole body of soft tissues), the assay is normally carried out using the hepatopancreas as the analytical sample, assuming, with reference to abundant previous data on toxin distribution in tissues, that most DSP toxins are accumulated in the digestive tissues. Although the digestive tissues (primarily hepatopancreas) accumulate most of the DSP toxins, a certain amount can be retained in other soft tissues (gill, mantle, siphon, gonads, etc.). When considering the percentage of hepatopancreas to whole shellfish soft tissues, significant inter- and intra-specific differences are evident, and the percentage will also depend on the season and location for a given species (hepatopancreas percentage variations of between 7% and 25% are routinely observed). Fig. 13.3 shows the DSP toxicity results expressed in relation to whole shellfish body weight of soft tissues that corresponds to the different survival times used as the toxicity criterion. These data are based on the assumption that when mice are injected with an extract equivalent to 5 g of hepatopancreas, a survival time of 24 hours corresponds to 0.8 mg OAeq kg^{-1} and a survival time of 5 hours corresponds to 2 mg OAeq kg^{-1}. By

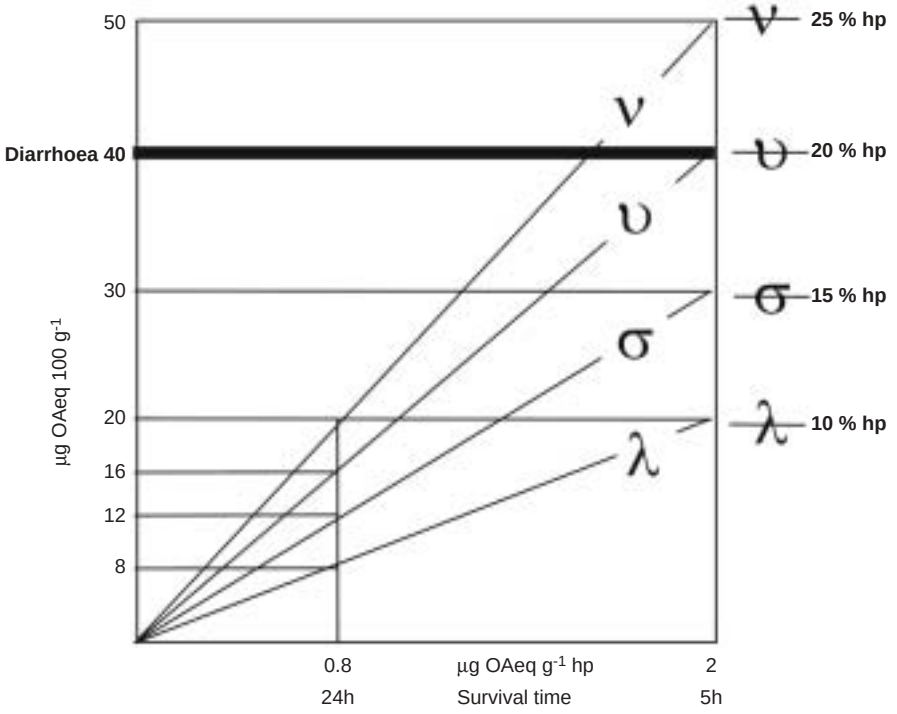

Figure 13.3
DSP toxicity (µg OAeq $100g^{-1}$ whole body weight of soft shellfish tissues) in relation to mouse survival time after intraperitoneal (i.p.) injection for different percentages of hepatopancreas (% hp) of whole body soft tissues. The onset of diarrhoea in humans would typically occur after the ingestion of about 40 µg OAeq.
Source: adapted from Miguez at al. (1998).

introducing different possible percentages of hepatopancreas in relation to whole shellfish soft tissue weight, different results are obtained. If a range of between 10% and 25% is considered, a survival time of 24 hours would correspond to a toxicity between 80 µg OAeq kg^{-1} and 200 µg OAeq kg^{-1} whole shellfish soft tissue, while a 5 h survival time would correspond to a toxicity value of between 200 µg OAeq kg^{-1} and 500 µg OAeq kg^{-1}, concentrations very close to or even higher than those reported to produce diarrhoea (total dose of 40–48 µg OA).

Clearly, when DSP toxins other than OA, DTX1 and DTX2 are present in shellfish, the time/dose response may vary, and a delay in the appearance of the symptoms may occur. This is the case for DTX3, for which an observation time of 24 hours is required (Terao *et al.*, 1993). Therefore, although the use of a 5-hour mouse test for DSP toxicity may improve the accuracy of the assay, it also increases the likelihood of shellfish with toxic levels of DSP analogues being accepted and placed on the market. The EU decision 2002/225/EC on survival time establishes that death of two out of three mice within 24 hours after inoculation into each of them of an extract equivalent to 5 g of hepatopancreas, or 25 g of whole shellfish' is the appropriate criterion of a positive result.

The assays described in the subsequent section are used for DSP toxicity determination in different countries, but the range of substances detected as well as the sensitivity may differ among protocols. These facts should be taken into account when making a decision on which method to use. Albeit in worldwide use for many years, none of these procedures has been evaluated in a formal collaborative study and although information on detection limit is in some cases available, performance characteristics, such as accuracy and precision, are not fully known.

13.4.1.1 *Rat bioassay*

The rat bioassay (Kat, 1983) is an oral toxicity test in which the presence of DSP toxins is indicated by feeding pre-starved (for 24 hours) white female Wistar rats, *Rattus norvegicus* (100–120 g), 10 g of shellfish hepatopancreas. On the following day, the consistency (diarrhoeic or soft) of the rat faeces is scored: normal (0 or -), normal to soft (1 or +), soft (2 or ++), soft to diarrhoeic (3 or +++) and diarrhoeic (4 or ++++). Faeces consistency other than normal suggests the presence of DSP toxins in the sample. If the toxicity score is above 0, shellfish are usually considered as unsafe for human consumption. The detection limit of the assay is around 800 µg OAeq kg^{-1} of hepatopancreas.

The rat bioassay simulates the mode of human intoxication, and the symptoms exhibited by rats are similar to those resulting from ingestion of the diarrhoeagenic DSP toxins by humans. Therefore, the rat bioassay is a biologically 'realistic' model of acute toxicity, and presents the additional advantage of not involving extraction of toxins, thus avoiding the possibility of low recoveries.

There are, however, serious limitations associated with this bioassay procedure. At best, the assay is only semi-quantitative and the dynamic range is poor. Due to broad individual variation in sensitivity and symptomology among rats, the precision is also questionable, particularly when few animals are assayed (typically one to three rats). The assay is reasonably diagnostic for the presence of the diarrhoeagenic DSP components (OA and DTX analogues), but PTX and YTX are not detected in this method. As the assay method is not very specific, other diarrhoeic agents including intestinal pathogens associated with poor animal health could also complicate the interpretation of faeces consistency.

13.4.1.2 *Intraperitoneal mouse bioassays*

All the mouse bioassay protocols detailed in this section require prior extraction and concentration of the lipophilic components by liquid-liquid partitioning. An advantage of the extraction protocols based on liquid-liquid partitioning of toxin components is that by modifying the acetone volumes used for the initial extraction, the protocols can be applied to DSP toxin determination on whole shellfish soft tissues. This can be very useful in the case of small shellfish species or when extracting eviscerated specimens. The acetone volumes required may vary depending on the shellfish species, but 100 g of homogenized whole shellfish tissues are usually used as the minimum test portion. When using whole shellfish soft tissues, the accuracy of the assay improves. However, the high volumes of organic solvents used, the laboriousness of the procedure and the toxicity of the solvents required for partitioning have previously limited the wide application of this approach in monitoring programmes involving the assay of a large number of samples. The recent inclusion of azaspiracid monitoring in toxin control programmes has renewed the importance of testing whole shellfish tissues (see Section 13.6.1 Azaspiracids). It is also worth mentioning that a mouse bioassay procedure based on extraction of whole soft tissues is used for NSP/DSP toxicity screening in New Zealand (described in Section 13.5.1.2). When inoculating mice with an extract corresponding to 25 g of whole soft tissues and using a 24-hour observation period, the detection limit of the assay is approximately 40 MU kg^{-1} or 160 µg OAeq kg^{-1}.

13.4.1.2.1 *Method 1*

The original mouse bioassay procedure of Yasumoto *et al*. (1978) was developed for bioassay-guided fractionation of toxic extracts, but it is widely applied for routine monitoring of DSP toxicity in shellfish. According to this method, a 20 g sample of homogenized hepatopancreas is extracted with 50 ml of acetone. The slurry is filtered into an evaporation flask and the tissue residue re-extracted twice with volumes of 50 ml of acetone and the filtrates combined. The solvent is removed by rotary evaporation under reduced pressure and the residue is re-suspended in 1% Tween 60 to a final concentration of 5 g equivalents ml^{-1}. One millilitre of this solution is injected intraperitoneally into each of three mice weighing between 18 g and 20 g, the mice observed for 24–48 hours, and toxicity determined on the basis of the survival time. The calculation of mouse units may require serial dilution of sample extracts until the minimum dose that produces death in 24 hours is reached. Under the conditions described here, the detection limit of the assay is 0.2 MU g^{-1} or 800 µg OAeq kg^{-1} hepatopancreas.

This procedure detects the presence of OA and DTXs, as well as the PTX and YTX groups. In addition, other lipophilic toxins, such as spirolides, gymnodimine, azaspiracids, brevetoxins and ciguatoxins are co-extracted, if present in shellfish. Mouse symptoms and death times may be similar depending on the bioactive compounds present in the samples, and in some circumstances it may be difficult to discriminate which substances are the cause of death of the mice.

A potential pitfall of this bioassay procedure is associated with the aqueous-lipid residue obtained after solvent evaporation. Depending on factors such as water content of the shellfish, re-suspension of the final residue to achieve a specific concentration (5 g hepatopancreas ml^{-1} Tween 60) may be inconsistent, thereby negatively affecting the accuracy of the assay. The high temperatures sometimes required for the total removal of the water may also produce low toxin recoveries (Croci *et al*., 1994).

Interference by low levels of PSP and ASP toxins, even at concentrations undetectable by the AOAC (1990) mouse bioassay, can also present problems. Some of these polar components can be co-extracted along with DSP toxins by acetone extraction, and they are concentrated during the evaporation procedure, yielding a final extract that can be highly toxic to mice. Fast mouse deaths (minutes to a few hours) with neurotoxic symptomology do not permit the prolonged observations required for DSP toxin detection. The presence of certain free fatty acids can also interfere with this assay (Takagi *et al*., 1984).

Among all the bioassays for DSP detection, this method is the least selective, and when positive results are obtained, it is not always feasible to assign the response to the presence of known toxins. However, after many years of routine application throughout the world, it has been proven to be reasonably effective in screening for the presence of DSP and other polyether toxins, and for public health protection.

13.4.1.2.2 *Method 2*

In this modified method (Yasumoto *et al*., 1984), a 20 g sample of homogenized hepatopancreas is extracted thrice with 50 ml of acetone. The extracts are filtered, then the filtrate is collected and the solvent removed by rotary evaporation. The residue is made up to 15–20 ml with water and the suspension is extracted thrice with 50 ml of diethyl-ether. The combined organic layers are backwashed twice with small quantities of water and evaporated to dryness. As in the original procedure described in method 1, the residue is re-suspended in 1% Tween 60 to a concentration of 5 g equivalents hepatopancreas ml^{-1} Tween 60 prior to i.p. injection. Toxicity is determined on the basis of the survival time or in mouse units.

The assay is very suitable for use with a wide variety of shellfish tissues. With this procedure, possible interferences with PSP and ASP toxins are removed in the water layer. High amounts of salts that are concentrated during the evaporation step, and could cause artefactual mouse deaths, can also be removed during the water-ether partitioning step.

The YTX components are partially solubilized in diethyl-ether; thus the recovery efficiency depends on the pH and lipid content of the sample. In spite of this drawback, the extraction method and assay are good enough to detect the presence of YTX at levels far below the maximum level regulated in the EU Decision 2002/225/EC (1 mg YTXeq kg^{-1}). A substantial improvement in YTX recovery is achieved if dichloromethane or ethyl acetate is used instead of diethyl-ether.

13.4.1.2.3 *Method 3*

In this procedure (Marcaillou-Le Baut and Masselin, 1990), hepatopancreas (20 g) is extracted thrice with 100% acetone and the filtrates collected. After evaporation of the acetone, the residue is re-suspended in 25 ml of methanol-water (80:20). The methanolic phase is washed twice with 25 ml of hexane, the hexane phases are discarded, and the methanolic layer is evaporated to dryness. The re-suspension of the residue in 1% Tween 60 and i.p. injection protocols are identical to those described for methods 1, 2 and 3. One of the main advantages of this assay is that low-polarity free fatty acids that could interfere with the assay are removed by the hexane wash. The final residue has a relatively low lipid content and is thus easier to accurately re-suspend in Tween 60, resulting in fewer false positive responses.

The main deficiency of the procedure is that the hexane washing step, besides removing free fatty acids, also preferentially solubilizes DTX3, the acyl-derivatives

of OA and DTX. If these components are present in shellfish samples, substantial loss of toxins can occur (Fernández *et al*., 1996). PSP toxins and interfering salts are not removed in this procedure.

13.4.1.3 *Suckling mouse bioassay*

The suckling mouse bioassay, a method usually applied for detection of bacterial enterotoxins, has been used to test the enteropathogenicity of OA, DTX1 and DTX3 (Hamano *et al*., 1986). The procedure involves the intragastric introduction *via* Teflon tubing of 0.1 ml of shellfish extracts suspended in 1% Tween 60, to four- to five-day-old mice. After the treatment, the mice are kept at 25°C for 4 hours then sacrificed with chloroform. The whole intestine is removed and the fluid accumulation (FA) ratio is determined as the ratio of the weight of intestine to that of the remaining body: FA = gut weight/(body weight - gut weight).

The detection limit of the test is lower than that of the i.p. mouse bioassay, and positive results can be observed at a level of 0.1 MU, corresponding to an FA value of 0.09. Pectenotoxins, YTXs and free fatty acids are not detected by this procedure. Although under optimal conditions results can be obtained within 4 hours, with a sensitivity higher that that of the mouse assay, accuracy and precision is a concern and the assay is difficult to be applied routinely. For example, wounding of the mice can take place during administration of the sample.

13.4.1.4 *Daphnia magna bioassay*

Daphnia magna is a freshwater cladoceran invertebrate traditionally used as a standard ecotoxicological indicator organism for the testing of different toxicants. A *D. magna* bioassay was developed by Vernoux *et al*. (1993) for OA screening of mussel extracts, based on the measurement of the inhibition of *Daphnia* mobility due to the presence of OA. The test is carried out in glass tubes each containing 10 ml of growth medium and five *Daphnia* individuals. Standardized culture conditions are crucial to avoid variability of responses. A linear correlation ($r = 0.74$) has been established between results of the *Daphnia* bioassay and an HPLC-FD method involving pre-column derivatization. As a larger number of animals can be used, precision can potentially be higher than that of the mouse bioassay, and sensitivity is 10 times higher. In addition to OA, other co-extracted lipophilic toxins may be detected. Although the method was proposed as an alternative to the mouse assay for OA screening, there have not been further reports on its application. It is therefore difficult to assess its value as a tool for routine DSP toxin monitoring.

13.5 Neurotoxic shellfish poisoning (NSP) toxins

The toxins implicated in neurotoxic shellfish poisoning (NSP), known collectively as ‘brevetoxins’, are considered to be primarily ichthyotoxins. In humans, the symptoms of NSP intoxication include respiratory distress, as well as eye and nasal membrane irritation, caused principally by exposure to sea-spray aerosols and by direct contact with toxic blooms while swimming. The brevetoxins are also accumulated in shellfish, which when consumed by humans cause a toxic syndrome somewhat similar to PSP intoxication. Many of these lipid-soluble cyclic polyether compounds have been characterized, including brevetoxins A, B and C (BTX A, B and C) [synonyms: PbTx–1, PbTx-2 and PbTx-8, respectively], GB3, GB5 and GB6

[synonyms: PbTx-3, PbTx-4 and PbTx-5, respectively] and PbTx-6 and PbTx-7, but undoubtedly there exist other undescribed derivatives. Due to the chemical lability of the brevetoxins, analysis of these compounds continues to be problematic (see also Chapter 8). All of these derivatives exert their toxic effect by specific binding to site 5 of voltage-sensitive Na^+ channels, leading to channel activation at normal resting potential.

13.5.1 Bioassay protocols

13.5.1.1 *APHA protocol*

The official method for the determination of NSP toxins is the American Public Health Association (APHA, 1985) procedure (originally Irwin, 1970), based on a diethyl-ether extraction of shellfish tissue. The APHA protocol for NSP is used extensively in the USA, where the problem of NSP is most prevalent and acute.

Apparatus

- electric blender;
- analytical balance;
- hotplate
- explosion-proof centrifuge with 250 ml centrifuge cups; covered with foil;
- explosion-proof chemical hood;
- 1,000 ml separatory funnel
- 400 ml beakers;
- disposable syringes with 26-gauge needles (do not reuse);
- stopwatch, mechanical or electronic, registering to at least 1 s.

Reagents

- hydrochloric acid (HCl), concentrated;
- sodium chloride (NaCl);
- cottonseed oil;
- diethyl-ether, anhydrous, AR (ACS). Use ether only from a previously unopened container – peroxides reduce apparent toxicity.

Test animals

Healthy albino male mice (Swiss-Webster strain) weighing 20 ± 1 g are preferable, however, mice weighing between 15 g and 25 g may be used. Do not reuse surviving mice. As mice often eat excessively and may store 1–2 g of food in the digestive tract, they should be fed sufficiently but not overfed.

Sample preparation and extraction

1. Clean, remove shell and drain shellfish. The number of shellfish required for 100 g of homogenate varies from two large clams to eight to ten small oysters. Homogenize shellfish tissues in an electric blender at high speed for 5 min. Weigh 100 g of homogenate into a pre-weighed 400 ml beaker and add 5 g NaCl and 1 ml concentrated HCl. Stir well. Heat mixture to boiling and cook for 5 min; stir frequently. Cool to room temperature and transfer to a 1,000 ml separatory funnel. Rinse beaker with ether and add rinse to separatory funnel. Perform all subsequent steps under an explosion-proof hood.
2. Add 100 ml ether to homogenate, stopper, and shake vigorously (venting frequently) for 5 min. Centrifuge at 2,000 × g for 15 min. Carefully decant upper

clear yellow ether phase into a 1,000 ml separatory funnel, keeping solids in the centrifuge bottle. Repeat extraction thrice more until the total amount of ether used is 400 ml. Drain off and discard any bottom layer containing small shellfish pieces and/or water emulsion so that only the ether phase remains. Transfer ether extract to a 400 ml beaker pre-weighed to the nearest 0.01 g. Allow ether to evaporate in air under the hood until no trace of ether fumes is discernible. An oily residue, which is the crude toxin extract, will remain. Cover tightly and freeze for later bioassay.

Mouse assay

Bring weight of crude toxin residue to 9.17 g with cottonseed oil. The volume of oil and toxin mixture represents 10 ml. Thoroughly mix and break up remaining pieces of insoluble matter as much as possible with a stirring rod.

Slowly fill syringe (with needle in place) with 1 ml of residue cottonseed oil mixture. Carefully inject (i.p.) 1 ml into each of two weighed mice on the ventral side anterior to the hind leg. If more than one drop of injected mixture leaks from the mouse, reject the mouse and inject another. Record time of injection. If the two mice survive for 2 hours, inject three more mice with 1 ml of residue cottonseed oil mixture or if the two mice die in less than 2 hours, make dilutions until the injection solution causes the death of two mice in 2–6 hours. [*Note: the recommended dilution is 1:1.25, made by adding 2 ml cottonseed oil to the remaining 8 ml of residue cottonseed oil mixture.*] Repeat dilutions if necessary. When the correct dilution is found, inject three more mice.

Observe mice continuously for 6 hours. The death time is the time elapsed from injection to the last breath of the mouse. The eyes will darken immediately on death. If mice survive for 6 hours, hold them for a total of 24 hours. For a 6-hour continual observation period, the lower limit of the assay sensitivity is 200 MU kg^{-1} shellfish tissue. Extending the continuous observation period to 15.5 hours will increase the assay sensitivity to 100 MU kg^{-1} shellfish. If mice die following continuous observation but within the 24-hour period, toxin is present in low quantity.

Death may not occur during an assay, yet physiological signs of NSP toxicity may be observed. The common signs of low NSP toxicity (non-lethal) are weakness of the limbs, imbalance, occasional respiratory spasms, and prolonged lethargy. Acute toxic signs include front and hindquarter paralysis resulting in instability, laboured breathing, prostration, or hyperactivity. To observe non-toxic behaviour for comparison, inject two mice with only cottonseed oil to serve as controls. The relationship of dose to death time of mice injected i.p with NSP toxins, along with weight correction factors, is presented in Table 13.4.

Toxicity calculations

Using Table 13.4, calculate the corrected MU activity using the following formula: MU × weight correction × dilution × 10 = MU kg^{-1} shellfish. If additional dilutions were not made, use the 10-fold dilution factor – based on the initial addition of cottonseed oil to 10 ml. If additional dilutions were made, multiply these factors also. Interpolate death times and weight corrections that fall between table values. *Example*: If a 22.3 g mouse died after 390 min, using the original dilution, then MU $kg^{-1} = 1.9 \times 1.14 \times 10 \times 10 = 217$.

TABLE 13.4 Relationship of dose to death time and weight of mice injected i.p. with NSP toxins (brevetoxins) extracted from shellfish

Death time (min) (20 g mice)	Mouse units (MU ml^{-1})	Mouse weight (g)	Weight correction factor
8	10.0	15	0.69
10	9.0	16	0.75
12	8.0	17	0.81
14	7.0	18	0.87
16	6.0	19	0.94
18	5.0	20	1.00
20	4.5	21	1.06
30	4.0	22	1.12
38	3.8	23	1.18
45	3.6	24	1.24
60	3.4	25	1.30
83	3.2	26	1.36
105	3.0		
140	2.8		
180	2.6		
234	2.4		
300	2.2		
360	2.0		
435	1.8		
540	1.6		
645	1.4		
780	1.2		
930	1.0		

Source: APHA (1985).

If mice die after continuous observation, calculate MU as though death occurred at the end of the continuous observation period. Report results as an indeterminate value of 'less than' (<), i.e. less than the sensitivity of the test for that period. If mice survive the 24-hour period, assign a value of <10 MU, because the lowest, reproducible sensitivity of the assay is 100 MU kg^{-1} shellfish meat.

Calculate the mean MU if 100% mortality occurs and death times are determinate, or determine the median MU if less than 100% mortality is observed or if death times are indeterminate. When reporting indeterminate toxicity, note the number of mice that died in 24 hours, or if no mice die in 24 hours, report toxin as undetectable (<100 MU kg^{-1} shellfish meat). Consider any detectable level of toxin per kilogram shellfish tissue as rendering the shellfish potentially unsafe for human consumption.

13.5.1.2 *New Zealand protocol*

After the emergence of NSP in New Zealand in 1993, a management strategy to monitor these toxins together with DSP toxins was developed by the Ministry of Agriculture and Forestry (MAF) Regulatory Authority. The sample preparation method used for the screening of NSP and DSP toxins is based on acetone extraction

of these lipophilic components, followed by partitioning into dichloromethane (Hannah *et al*., 1995). Death of two of three mice within 6 hours is considered to be indicative of the presence of NSP or DSP toxins. In this protocol, the presence of DSP toxin is confirmed using an ELISA test and NSP levels are confirmed by the official APHA method, to avoid interferences due to gymnodimine (see Section 13.6.3).

Materials and equipment

- high-speed flameproof blender;
- blender cups and lids;
- Whatman 541 filter paper;
- cotton wool;
- round-bottom flasks – 500 ml and 1,000 ml;
- separating funnels – 500 ml;
- rotary evaporator;
- Buchner funnels and flasks;
- glass vials – 20 ml;
- balance;
- hotplate;
- acetone, analytical reagent (AR) grade;
- dichloromethane (AR);
- sodium sulphate (anhydrous AR), heated in a muffle furnace at 400°C for 16 h;
- sterile Tween 60/Saline, 1%:0.85%, sterilized by autoclaving;
- healthy mice weighing 20 ± 2 g;
- syringes with 26- to 23-gauge needles;
- stopwatch;
- coloured paints/markers for coding mice.

Extraction procedure

1. Blend 100 g of shellfish soft tissues at high speed for 60–120 s until homogeneous. Sample homogenates shall be extracted within 2 hours of blending. Add 300 ml acetone and homogenize for 20 s. Filter the extract through filter paper (Whatman 541) using a Buchner funnel into a 1,000 ml round-bottom flask. Return the solid residue to the blender cup. Add 200 ml acetone and homogenize for 20 s. Filter as before into the same flask. Filter combined extracts through cotton wool to remove any solid material that has precipitated from the extract on contact with the cold glassware.
2. Remove acetone by rotary evaporation at 35 ± 2 °C. *Extracts may be covered and refrigerated at this point if overnight holding is necessary.* Add 100 ml dichloromethane to the aqueous-lipid residue and transfer the mixture to a 500 ml separating funnel. *Gently* shake the funnel and allow layers to separate. *Vigorous shaking may create toxin-containing emulsions at the interface.* Draw lower layer through anhydrous Na_2SO_4 (pre-rinsed with dichloromethane) into a 500 ml round-bottom flask. (*This step is to remove any remaining water from the extract*.) Add 80 ml dichloromethane to the funnel. *Vigorously* shake the funnel and allow layers to separate. Drain the lower layer through anhydrous Na_2SO_4 into the same flask. Wash through anhydrous Na_2SO_4 with 20 ml dichloromethane and collect in the same flask. Remove dichloromethane by rotary evaporation at 35 ± 2°C. Transfer the residue, with minimum volume dichloromethane, to a 20 ml glass vial. Remove dichloromethane by rotary

evaporation or under a stream of nitrogen on a warm (40°C) hotplate in a fume hood, and then by rotary evaporation to a constant weight (±5 mg).

3. Accurately weigh the residue and re-suspend in Tween 60/saline such that each millilitre of final volume contains the equivalent of 20 g of shellfish tissue. For a 100 g sample, the final volume required is 5 ml. The weight of Tween/saline required is determined as follows: 5.00 – volume of lipid extract, where volume of lipid extract = weight of lipid/0.917. If cottonseed oil is used, the final weight = 4.59 g. [*Note: The use of cottonseed oil is optional for samples that are difficult to suspend in Tween 60/saline. The density of extracted lipid is assumed to be 0.917 as per recommended procedures. However, changes in extraction solvent may result in different lipid extracts with different densities. This should be considered if anomalous results are encountered.*]

Mouse bioassay for DSP/NSP toxicity

Colour-code each of three mice in order to accurately keep track of elapsed times. Inoculate 1 ml of 20 g ml^{-1} intraperitoneally into each of three mice (wt 18–22 g). Note and record the time of inoculation to the nearest 5 s. Observe continuously for 6 h. [*In practice this means that the mice must be monitored every few minutes so that a reasonably accurate time of death may be noted*.] The time of death is determined by the last gasping breath. Assayists should note the manner of mouse death. This could provide useful information if non-NSP toxins are encountered. Record the time of death and calculate death times for each mouse. Continue observation for 24 h.

Interpretation

Two or more deaths within 6 hours	Suspect NSP (>200 MU kg^{-1}). Confirm NSP using ether extraction (APHA method). Check for DSP toxins using ELISA
One or no deaths in 6 hours	NSP not detected (<200 MU kg^{-1})
Any deaths in 6–24 hours	Check for DSP toxins using ELISA
No deaths in further 18 hours	DSP not detected

13.6 Cyclic imine/amine shellfish toxins

13.6.1 Azaspiracids

Azaspiracids (AZAs) are a recently identified group of shellfish toxins, provisionally associated with the dinoflagellate *Protoperidinium crassipes* (Yasumoto, pers. comm.), that are responsible for an emerging type of shellfish intoxication in Europe (named azaspiracid poisoning (AZP) (Ofuji *et al*., 1999*a*, 1999*b*)). Cases of this toxic syndrome were first reported in the Netherlands in November 1995, caused by the ingestion of mussels from Killary Harbour, on the west coast of Ireland (McMahon and Silke, 1996; Satake *et al*., 1998). Acetone extracts of mussel samples from this harvest area gave strongly positive results following i.p. injection in mice and oral feeding of hepatopancreas to rats. Mice showed aberrant neurological symptoms,

such as respiratory difficulties, spasms, slow progressive paralysis of the limbs and, at higher doses, death within 20 min, clearly differing from those of DSP toxicity.

Human symptoms caused by AZA toxins are similar to those caused by OA and DTXs, and include nausea, vomiting, severe diarrhoea and stomach cramps. Neurological disorders have not been reported. *In vivo* studies with mice have shown that the target organs of AZAs are the digestive tract, liver, pancreas, thymus and spleen (Ito *et al*. 1998, 2000).

Azaspiracids are chemically characterized by a trispiro-assembly, a spiro-ring unique in nature fused with a 2,9 dioxabicyclo-nonane ring and a carboxylic acid (Satake *et al*., 1998) (see Chapter 8). In addition to the original azaspiracid structure (AZA1), four analogues have been characterized: AZA2 (8-methylazaspiracid), AZA3 (22-demethylazaspiracid) (Ofuji *et al*., 1999*a*), AZA4 (3 hydroxy-22-demethyazaspiracid) and AZA5 (23–hydroxy-22-demethyl-azaspiracid) (Ofuji *et al*., 2001). The chemical structure of AZA4 and AZA5 suggests that they are oxidized metabolites of AZA3.

The anatomical distribution of AZAs in shellfish differs from that of DSP toxins, and the toxins can be readily disseminated from the digestive tract into other tissues. Whole shellfish tissues should therefore be considered as potentially toxic and included in the assay sample.

13.6.1.1 *Bioassay protocols*

13.6.1.1.1 *Mouse bioassay*

Recent findings on the tissue distribution of AZAs have led to the reconsideration of the applicability of the existing mouse bioassay procedures for polyether toxins. The mouse bioassay detailed in method 1 (Section 13.4.1.2.1) (Yasumoto *et al*., 1978) may be used for the detection of AZA in shellfish, but because only the hepatopancreas is extracted in this procedure, and AZA is distributed throughout the entire shellfish soft tissues, a significant amount would not be taken into account. A bioassay using an extract of whole shellfish tissue seems more appropriate. A specific mouse bioassay procedure has been developed for AZA; 100% acetone can be used for the initial extraction of fresh whole shellfish, followed by a further extraction of tissues with methanol (twice) and liquid-liquid partitioning with diethyl-ether after evaporation of the extracting solvents (Yasumoto *et al.*, in prep.). The lower limits of detection, using a 24 h observation period, is reported to be 2–4 µg AZA per 20 g mouse (Ofuji *et al*., 2001). If mice are inoculated with an extract corresponding to 25 g, the detection limit would correspond to 80 to 160 µg AZA kg^{-1} shellfish tissue.

13.6.1.1.2 *Rat bioassay*

Azaspiracids produce diarrhoeic effects, and therefore (in principle) the rat bioassay outlined in Section 13.4.1.1 can be used for their detection. Unfortunately, given the absence of quantitative standards for toxicity testing, the detection limit of this oral test has not yet been determined.

13.6.2 Spirolides

In 1991, routine biotoxin monitoring of bivalve molluscs for DSP toxins at aquaculture sites along the eastern shore of Nova Scotia, Canada, revealed a novel and

highly potent toxic response in mice after i.p. injections of lipophilic extracts. The symptoms, which included rapid deaths and were usually preceded by apparent neurological symptoms, were very different from those associated with known shellfish toxins, including those responsible for DSP or PSP intoxication. The symptoms of this novel 'fast-acting toxin' in mice, after i.p. administration of contaminated shellfish extracts, vary depending on survival times and may include piloerection, splaying of the back legs, ataxia, ophthalmia, abdominal muscle spasms, hyperextensions of the back, tail whipping and arching towards the head, and immediately prior to death, front leg tremor, extension of the hind legs and loss of bladder control (Richard, unpublished data). Extremely high doses tend to produce non-specific symptoms while survival times approaching the LD_{50} produce more specific symptomology. Elevated levels of spirolides in shellfish have not been categorically linked to any human intoxication.

A group of cyclic imines named spirolides, consisting of a spiro-linked tricyclic ether ring system and an unusual seven-membered spiro-linked cyclic iminium moiety (see Chapter 8 for structures), were isolated from shellfish and later plankton samples (Hu *et al*., 1995, 1996, 2001) and, eventually, the causative organism was identified as the dinoflagellate *Alexandrium ostenfeldii* (Cembella *et al*., 2000). Spirolides belonging to groups A, B, C and D are biologically active, whereas spirolides E and F, isolated only from shellfish (Hu *et al*., 1996), are not. Biological inactivity of the latter compounds, which may be degradation metabolites, is presumably due to the opening of the cyclic imine ring.

In toxicological studies, the oral and intraperitoneal toxicities of spirolides in mice were determined to be approximately 1 mg kg^{-1} and 40 µg kg^{-1}, respectively, for a lethal dose comprising mostly desmethyl-spirolide C (Richard *et al*., 2001) (Fig. 13.4). The pharmacological effects of spirolides were also characterized by subjecting mice to various drugs (e.g. atropine, physostigmine, propanolol and epinephrine), followed by a challenge of a spirolide-rich extract of *A. ostenfeldii* cultures. The results suggested that spirolides affect the muscarinic acetylcholine receptors in mammalian systems, however this was not confirmed in further initial studies with specific receptor assays. Recent neuropathological studies with rodents exposed to purified spirolides (Pulido *et al*., 2001) have shown evidence that the hippocampus and brain stem are possible target regions in mice. At the transcriptional level, the early injury markers (EIM)-*Hsp*72, *c-fos*, and *c-jun*, and the muscarinic acetylcholine receptors were altered between control and spirolide-challenged animals.

Spirolides are not yet considered to be of concern to shellfish consumers, therefore no regulatory protocol or action levels for closure of shellfish harvesting have been established. Nevertheless, when detected by the DSP mouse bioassay, shellfish producers have voluntarily withheld shellfish from the market until the unusual mouse toxicities associated with spirolides have abated.

13.6.2.1 *Mouse bioassay protocol*

As is the case for gymnodimine and certain other 'fast-acting toxins' of dinoflagellate origin, the recognition of characteristic symptoms of spirolide toxicity in mice first arose as a result of routine bioassays directed at other lipophilic toxins. Thus, the use of conventional extraction and preparation protocols for DSP toxins, such as the acetone extraction procedure (Yasumoto *et al*., 1978) described in Section 13.4.1.2.1 (Method 1), are adequate for extraction of spirolides and no specific modifications

are required in the bioassay. Due to the highly acute toxicity of spirolides administered intraperitoneally into mice and the specific symptomology, the mouse bioassay is an effective method of monitoring spirolide toxicity in shellfish. The survival time of i.p. injected mice is typically 3–20 min and is often, but not always, preceded by neurological symptoms, including convulsions. If a mouse survives past 20 min, even while demonstrating symptoms, it will normally recover fully and quickly. When extracts produce symptoms typical for spirolides, and other lipophilic toxins are not confirmed by analytical methods such as LC-MS, to be present, interpretation of the bioassay is rather straightforward. When using the mouse bioassay, the synergistic or antagonistic effects due to the presence of other co-extracted lipophilic toxins in shellfish matrices remains undetermined. At the very least, the presence of spirolides may mask the presence of DSP toxicity, and in turn the interpretation of mouse bioassay results, for which long observation periods are required and extended death times are common.

13.6.3 Gymnodimine

In early 1994, intraperitoneal toxicity in mice was detected during the analysis of shellfish extracts prepared by the New Zealand Health Department using the acetone extraction method (Yasumoto *et al*., 1978), followed by dichloromethane partition (Hannah *et al*., 1995). Dredge oysters (*Tiostrea chilensis*), in particular, were found to be highly toxic, but further analysis did not demonstrate any detectable level of

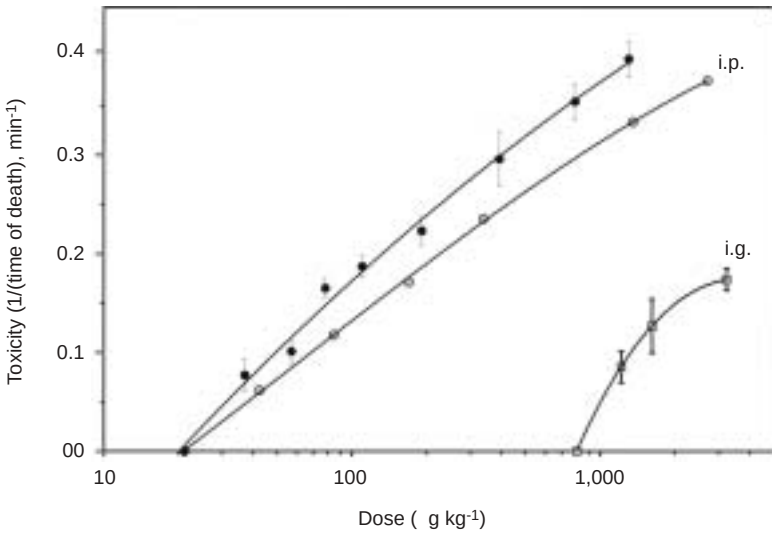

Figure 13.4
Toxicity of spirolides in mice through i.p. and oral (i.g.) dosing. The curves are logistic dose-response curve fits (SigmaPlot). The i.p. data points are means of duplicate tests, whereas those for the i.g. tests are means of 6–8 replicate tests, with standard deviations shown as error bars.
Source: adapted from Richard *et al*. (2001) and Richard, Quilliam and Cembella, unpublished data).

DSP or NSP toxins. Furthermore, the symptoms and dose response in mice did not match other known marine toxins. Undiluted shellfish extracts fed to rats and mice elicited no signs of oral toxicity (MacKenzie *et al*., 1996).

Isolation and purification of the novel toxin gymnodimine, a pentacyclic compound incorporating a C24 carboxylic acid and a fused azine (see Chapter 8), was accomplished with reference to a bioassay-guided fractionation of toxic shellfish (MacKenzie, 1994; Seki *et al*., 1995, 1996; MacKenzie *et al*., 1996). Gymnodimine is stable in acidic solutions but labile in basic solutions (Miles *et al*., 1999). The mode of action of the 'fast-acting toxicity' of gymnodimine has not been clearly elucidated, but symptoms are strongly characteristic of neurotoxicity. It should be noted that gymnodimine shares a high degree of structural homology with spirolides and pinnatoxin (see Chapter 8), thus cleavage of the nitrogenous ring will lead to deactivation (Stewart *et al*., 1997). In early studies, intraperitoneal mouse lethality (ddY strain) for gymnodimine was found to be 450 µg kg^{-1} (Seki *et al*., 1995). Administration of strongly toxic extracts yielded rapid and non-specific mortality in mice. Death times between 5 min and 15 min produced neurological symptoms such as curled tail, jumping and paralysis (Seki *et al*., 1996). Yet more recent studies on gymnodimine indicate higher potency than originally believed, with i.p. mouse toxicity values of 96 µg kg^{-1} (Miles *et al*., 1999). Based on preliminary work indicating low oral potency in rodents (MacKenzie *et al*., 1996), and after a one-year closure of areas affected by gymnodimine, the New Zealand Marine Biotoxin Management Board made the decision that in cases where only gymnodimine was detected further shellfish area closures were not required. While there has been no indication that gymnodimine has any effects on humans, the New Zealand Marine Biotoxin Technical Committee has decided that more formal toxicity studies should be conducted under the protocols of the Organisation for Economic Co-operation and Development (Paris).

13.6.3.1 *Mouse bioassay protocol*

As gymnodimine is readily extracted by the Yasumoto protocol for DSP toxins (Section 13.4.2.2.1), it is presently considered to be an undesirable artefact of this DSP toxin extraction procedure. No specific mouse bioassay procedure has been developed for the monitoring of this compound in shellfish tissues. During the original investigative phase, it was determined that the ether extraction protocol had the effect of reducing the toxicity of shellfish extracts due to gymnodimine by greater than 90% (MacKenzie *et al*., 1996). Confirmation of gymnodimine as the cause of a positive mouse bioassay is most conveniently done using LC-MS methods (Stirling *et al*., 2002; see Chapter 8). Partitioning an acidified acetone extract between water and ethyl acetate (in place of dichloromethane) can also reduce the amount of gymnodimine detected in the mouse bioassay (Yasumoto, pers. comm.).

13.7 Conclusions

There is still a paucity of simple, rapid, robust and sensitive procedures that can be used with reliance by regulatory laboratories for public health control of seafood toxicity caused by phycotoxins. This is particularly true for the toxins associated with PSP, DSP, NSP and for ciguatera poisoning. In the near future, certain *in vitro* assays (see Chapter 12) may be applied as routine screening tools for regulatory

purposes, but to date, in spite of increasing ethical considerations, most countries rely primarily or exclusively on mammalian bioassays. In some countries, regulations against the use of experimental animals may complicate effective phycotoxin monitoring, as alternative toxicological procedures are not readily available. In any case, for the foreseeable future both regulatory regimes and toxicological researchers will probably remain dependent on effective whole-animal bioassays for screening of acute toxicity effects and for studies on the effects of chronic toxin exposure.

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Part II

Taxonomy

14

Taxonomic principles

G. M. Hallegraeff

Harmful algal blooms are often almost monospecific events. Correctly assessing the precise taxonomic identity of the causative organism thus becomes crucial in deciding whether knowledge on toxicology, physiology and ecology gained from similar blooms elsewhere can be reliably applied to the species at hand. Resolution of the species concept in harmful algae has become a profound issue of discussion at all major conferences dealing with toxic phytoplankton.

14.1 What is a species?

While genera are more or less subjective taxonomic units that attempt to reflect close relationships, species are supposed to be evolving biological units (see Taylor, 1993, for a discussion of the history of development of this concept). This has been the basis for the Biological Species Concept with species boundaries defined according to the ability of organisms to interbreed and produce viable offspring. While readily applicable to Metazoan animals, there are problems with plants where some apparently distinct species successfully hybridize (often with weakly viable offspring) and in particular with protists for which sexual fusion is infrequent or apparently absent. For dinoflagellates, for example, sexuality has been documented for only about 10% of species (see Chapters 15 and 20) and accordingly very few attempts have been made to use sexual interbreeding for strain and species definition (see Beam and Himes, 1982, for *Crypthecodinium cohnii*; Blackburn *et al.*, 2001, for *Gymnodinium catenatum*; Anderson *et al.*, 1994, for *Alexandrium tamarense*). These studies have revealed the existence of morphologically similar natural populations that are reproductively isolated, analogous to the 'sibling species' concept known from animal studies. For most harmful algal species, however, the usual mode of proliferation is by asexual fission and sexuality in addition to genetic recombination serves a special purpose in the life cycle of these organisms (cyst formation and survival in dinoflagellates; auxospore formation and cell enlargement in diatoms). For permanently asexual organisms, only discontinuities in morphological or biochemical characters can be used to constitute species boundaries.

14.2 Taxonomic criteria used for identification and classification

Morphology of an organism is the complex expression of its genotype, subject to phenotypical change due to the environment, life-cycle transformations and other

influences. Morphological traits and biogeographical distributions of organisms continue to be considered as the primary means for traditional species classification. Through examination of thousands of individuals one needs to develop an understanding of what are conservative characters useful for taxonomy and what are highly variable characters. Cultured cells can have more variable morphology than field material and considerable care should be exercised in basing new species descriptions exclusively on cultured material. For diatoms useful conservative taxonomic features include strutted/labiate/occluded process patterns and diatom valve markings (number of striae; areolae in 10 µm) (Chapter 17). For armoured dinoflagellates thecal plate patterns are the most diagnostic, with hypothecal characters being more conservative than epithecal ones (Chapter 15). For less-ornate unarmoured dinoflagellates and especially nanoplanktonic and picoplanktonic taxa (such as *Aureococcus anophagefferens*) ultrastructure of chloroplasts, pyrenoids, flagellar roots, etc., have become indispensable morphological adjuncts. In addition, non-morphological characters such as lipid, pigment and toxin biochemistry, immunocytological traits, chromosome number and more recently nuclear or plastid DNA sequences are now being increasingly used to aid in species recognition (for example, see Daugbjerg *et al.*, 2000, for *Gymnodinium/Gyrodinium*). While marine phytoplankton species have remained morphologically conservative they can have accumulated significant genetic variability. Looking alike does not necessarily mean genetically identical and looking different does not mean genetically isolated (Taylor, 1993). Morphospecies designations therefore sometimes can be of limited use for ecological purposes. The dinoflagellate *Alexandrium tamarense* is known to exist as toxic and non-toxic strains, bioluminescent and non-bioluminescent populations, and cold-water and warm-water forms. In some cases the use of biochemical, molecular and physiological data has corroborated morphotaxonomy (e.g. arguments for the synonomy of *A. minutum* and *A. lusitanicum*; Costas *et al.*, 1995), while in other cases apparent conflicts have inspired a revaluation of traditional species discrimination (e.g. the *Phaeocystis pouchetii* complex; Medlin *et al.*, 1994; the elevation of *Pseudo-nitzschia multiseries* to species level; Manhart *et al.*, 1995) or provoked further debate on the species concept in unicellular algae. While the presence of a ventral pore in the first apical plate of the dinoflagellate *Alexandrium* has been widely accepted as a stable taxonomic character (Balech, 1995) which is used to discriminate *A. tamarense* from *A. fundyense*, Anderson *et al.* (1994) demonstrated that cultured clones of the two taxa were sexually compatible. Furthermore, Scholin and Anderson (1994), working with the same *A. tamarense* 'species complex', found that ribosomal RNA sequences of isolates clustered more logically on the basis of geographic origin than morphotaxonomy.

14.3 General comments about taxonomic nomenclature

The science of taxonomy seeks to delimit stable groups of individuals that share common traits. The correct name for a phytoplankton organism should fulfil stringent requirements as spelled out by the *International Code of Botanical Nomenclature* (*ICBN*) (most widely used for algae), *International Code of Zoological Nomenclature* (*ICZN*) (sometimes used for dinoflagellates and euglenoids which have a large proportion of colourless species) or the *International Code of Nomenclature of*

Bacteria (used for cyanobacteria). The aim of these codes is to produce nomenclatural stability. Under the *ICBN*, for species descriptions after 1 January 1958 there is a requirement for a written description of the essential characters, an illustration, a Latin diagnosis and a designation of type material. The name must not have been used previously in that rank for a member of the Plant Kingdom (if it had it would be referred to as a homonym) and it must have priority, being published before any other name applied within the same rank to the same organism (others being referred to as synonyms).

More and more, the practice of lodging permanent mounts in a museum or herbarium is being replaced by the designation of light or electron micrographs as holotypes, while with (cyano) bacteria pure cultures can serve as types (Stanier *et al.*, 1978). The common practice with bacteria of lodging molecular sequences in the GenBank is now increasingly applied also to new species descriptions of microalgae. The recommendation by the *ICBN* that names above the level of genus should be typified (e.g. the class Prymnesiophyceae based on the genus *Prymnesium)* has not been generally accepted (i.e. leaving the name Haptophyceae as a valid alternative, Chapter 16). The ending of latinized species names indicates the gender (masculine, feminine, neuter) and the species gender must agree with that of the genus and automatically be corrected if it does not. Hulburt thus made a mistake when naming a species of *Amphidinium* after the female English scientist Nellie Carter by calling it *carteri*, which had to be corrected to *carterae*. When new combinations result from transferring species from one genus to another, the species name should be retained, unless it has already been used with the genus to which the species is brought, in which case a new species name is provided. Since 1 January 1953, the basionym (the combination under which the species first appeared) must be cited, with publication details provided. An author's name in parentheses means that since the original description of the taxon its name has been changed. There are important procedural differences between the *ICBN* and *ICZN* (e.g. requirement or not for a Latin diagnosis), and neither the *ICBN* nor *ICZN* recognize as homonyms those names proposed under one code but preoccupied under the other. This can lead to absurd situations in which a scientist declaring him/herself a zoologist can be precluded from using names that a botanist can use (Patterson and Larsen, 1992). One example is the dinoflagellate genus *Phalacroma* created by Stein (1883) within the Plant Kingdom, but which to zoologists is preoccupied by the trilobite genus *Phalacroma* (Howle and Corda, 1847). A proposal (Taylor *et al.*, 1987) to resolve these nomenclatural problems was not accepted at the Berlin Botanical Congress. There are also differences between botanists and zoologists in the recognition of infraspecific categories. Botanists use the terms *variety* (often conceived as small differences in genotype) and *form* (a response of an organism with the same genotype to a different environment), although the meaning of these terms is not agreed upon, while zoologists only recognize the term *subspecies*, which carries with it a notion of geographic isolation and lack of interbreeding which is difficult to apply to marine phytoplankton with virtually unlimited dispersal options. Other widely used infraspecific biological categories are *strain* (a genetic group, usually a culture, which is slightly different from others in the same species) or *clone* (if the cells in the strains are identical due to asexual reproduction). The neutral term *isolate* can also be used for a culture.

Palaeontologists working with fossil dinoflagellate cysts by consensus have chosen to treat them as form genera under the *ICBN*. When a dinoflagellate taxon has both a fossil (e.g. *Polysphaeridium zoharyi*) and modern representation (*Pyrodinium*

bahamense; Chapter 20), then the name-carrier should preferably be the living organism, as this provides the most complete information (Wall and Dale, 1968). This would necessitate, however, the official conservation of, for example, the genus *Gonyaulax* Diesing (1866) against the older equivalent cyst name *Spiniferites* Mantell (1850). An attempt to produce a unified classification of living motile dinoflagellates and fossil dinoflagellate cyst taxa has been prepared by Fensome *et al.* (1993).

14.4 Name changes

Name changes always cause concern and confusion among non-specialists, but reflect the ever developing scientific understanding of natural relationships among organisms. Two examples are provided to illustrate the taxonomic principles described above.

The diatom *Pseudo-nitzschia australis* Frenguelli (1939) was initially classified by Hasle (1965) as a section within the genus *Nitzschia.* As the name *N. australis* was preoccupied, the organism was newly named *Nitzschia pseudoseriata* Hasle. Subsequently, following a revived interest in the toxin-producing *Nitzschia* species, it was decided to revive the genus name *Pseudo-nitzschia* for species with step-wise overlapping colonies (Chapter 17) thus necessitating a return to the name *P. australis.* Similarly, *Nitzschia pseudodelicatissima* Hasle became *Pseudo-nitzschia pseudodelicatissima* (Hasle) Hasle, and *Nitzschia pungens* f. *multiseries* Hasle, now considered a separate species (Hasle, 1995), became *Pseudo-nitzschia multiseries* (Hasle) Hasle.

Another example is the toxic dinoflagellate *Gonyaulax tamarensis* (Lebour, 1925) which, once it was recognized that its thecal-plate pattern (four apicals, no intercalaries) did not fit in the genus *Gonyaulax*, was relegated for a number of years to either the poorly defined genus *Alexandrium* (Halim, 1960), *Gessnerium* (Halim, 1967) (based on an erroneously optically reversed *Alexandrium*) or *Protogonyaulax* (based on absence of contact between the first apical plate homologue [1′] with the apical pore complex [Po] (Taylor, 1979). This confusion was eventually solved by a re-examination of material of the type species *Alexandrium minutum* from the type locality in Alexandria Harbour, Egypt (Balech, 1989), which revealed the variable nature of the contact between 1′ and Po and thus indicated that *Protogonyaulax* cannot be maintained (Chapter 15). The dinoflagellate organism in question now should be called *Alexandrium tamarense* (Lebour) Balech. To alleviate the problems of ever-changing taxonomy of harmful phytoplankton, it is recommended: (a) to study type-material or, if this is not available, collect and re-examine material from the type locality (e.g. see Balech, 1989, for *Alexandrium minutum*; Hansen *et al.*, 2000, for *Gymnodinium aureolum*); (b) establish and curate type-specimen collections using permanent mounts, photomicrographs, videotapes and preferably living cultures; and (c) incorporate life-cycle features such as cysts in species descriptions (Steidinger, 1990). Original names should be retained as much as possible until complete information is available on the existing available and valid genera.

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15

Taxonomy of harmful dinoflagellates

F. J. R. Taylor, Y. Fukuyo, J. Larsen and G. M. Hallegraeff

15.1 What are dinoflagellates?

This group of unicellular eukaryotic organisms are termed 'flagellates' because the majority, including the harmful species, can swim by means of a pair of flagella, one of which is ribbon-like and wavy, and the other more conventional and whip-like. They rotate as they swim, but so do most flagellates. They occur in both salt and freshwater and can be both planktonic (up in the water) or benthic (associated with the bottom). Approximately 50% of the species are photosynthetic (Taylor, 1987). The group is characterized by the flagellar arrangement and related cell morphology (grooves associated with the flagella: see below), and by features such as their nucleus, known as a dinokaryon (see Spector, 1984, and Taylor, 1987, for general references dealing with the group as a whole). The classification of the group, including its rich fossil representation, has been substantially revised (Fensome *et al.*, 1993).

15.2 General comments about harmful dinoflagellate species

As is evident from a glance at this chapter, dinoflagellates are the protist group with the largest number of harmful species. The great majority is photosynthetic (*Noctiluca* and some species of *Dinophysis* are the notable exceptions), for no obvious reason other than that they are more likely to be abundant than non-photosynthetic species and their behaviour makes them amenable to physical aggregation. Both thecate and athecate species can be harmful. Most are marine. Chain-formers seem to be overrepresented, also for no obvious reason, and quite a few are closely related (Taylor, 1985, 1993).

15.3 Identification texts dealing with harmful dinoflagellates

The need for assistance in identifying potentially harmful species has led to the creation of documents, of which this is the latest. As many of these species are cosmopolitan within their latitudinal range, both northern and southern hemispheres (Taylor, 1987), aids created for one area may be useful in another. The general text on marine dinoflagellate identification by Steidinger and Tangen (1996) is an invaluable resource that contains descriptions of numerous harmful species. An early

source created especially for harmful species recognition by aquaculturists is that by Gaines and Taylor (1986), but more lavishly published and illustrated versions have been produced by Larsen and Moestrup (1989), Fukuyo *et al.* (1990) building on the species sheets issued by a Japanese working group on red tides since the late 1970s, and Hallegraeff (1991, 2002). Tropical benthic species potentially involved in ciguatera were described by Fukuyo (1981). Balech has contributed many recent descriptions, notably those in his most recent monograph on *Alexandrium* (Balech, 1995). These works should be consulted for further detail in descriptions, illustrations, reference citations and regional information. General taxonomic matters are discussed in Chapter 14.

15.4 Taxonomic criteria used in harmful dinoflagellate identification and classification

A brief guide follows to the criteria and terminology used in dinoflagellate description. Cell size and shape are obvious features used. Cell contents have not been used much because all harmful species recognized so far, with the exception of *Noctiluca*, are photosynthetic. Surface ornamentation (pores, spines, ridges, etc.) is also used if present.

Essentially we can distinguish between those that lack a readily visible, multiplate cellulosic wall, the theca (hence called 'athecate' dinoflagellates) from those that possess a theca ('thecate' dinoflagellates). The terminology relating to each is described below. Another distinction that can be made is between the great majority of dinoflagellates which have distinctive flagella arising from the (ventral) side of the cell, referred to as the dinokont condition, from the one harmful genus, *Prorocentrum*, in which they both arise from the anterior end, referred to as desmokont.

In describing dinoflagellates orientational terminology is used. The end that is forward when the cells move is named the apical pole and the opposite is the antapical. Desmokonts are considered to be laterally flattened. In *Prorocentrum*, which is thecate, the flagella arise from a field of tiny platelets that have not been used much in taxonomy so far. This periflagellar area is asymmetrically excavated out of one of the large lateral plates, the right valve, the other being the left valve.

In dinokonts the side that the flagella arise from is the ventral side and the opposite side is dorsal, with the organism's left and right following zoological convention. These terms are also used in describing the side that the cell is being viewed from. The ventral view is usually the most characteristic, except in highly flattened species. When sending a photograph or drawing to an expert this view should not be omitted. The cells can usually be rolled over by carefully tapping on the coverslip or using a glass needle. Size is important, although it is not unusual to find a cell that exceeds the published range by a few micrometres. 'Length' is usually the apical/antapical distance and 'width' is the transdiameter (from left to right sides at its widest) but in dinophysoids they usually lie on their left or right sides and ventral views are rare. In this case 'width' is dorso-ventral distance.

Other features used in descriptions include the position of the girdle (cingulum) and whether it is displaced or not, i.e. whether the proximal and distal ends meet or are offset. In the latter case, if the left (proximal) side is more anterior the displacement is left-handed, or it is right-handed if the opposite is true. The former is much more

common. The degree of offset is given in girdle widths. In *Alexandrium*, for example, the girdle has a left-handed displacement of approximately one girdle width.

In thecate species the plate pattern, tabulation, is extremely important. Due to extensive study its degree of reliability is well established (some features are more conservative than others: see Taylor, 1987). The description of new species or any critical taxonomy requires complete elucidation of the plate pattern, which can be difficult, requiring special techniques which are explained in Appendix 15.1. Fortunately, for most routine identifications this is not necessary, identification relying on general form, size and one or two key characters. The descriptions below focus on these.

The system used most widely for dinoflagellate tabulation (Fig. 15.1) is known as the Kofoid System after its developer C. A. Kofoid, with subsequent modifications, particularly for the sulcal plates by E. Balech. While it is usually simple to apply there are problems, particularly when used for comparisons (discussed in detail by Fensome *et al.*, 1993). It is applied principally to gonyaulacoid (e.g. *Alexandrium*, *Gonyaulax*) and peridinioid (e.g. *Protoperidinium*) species. Essentially there are five primary latitudinal series (listed from the apical end): apicals, precingulars, cingulars, postcingulars and antapicals. Individual plates or incomplete series between these series, particularly the apicals and precingulars, are termed intercalaries, anterior if on the epitheca, posterior if on the hypotheca. A cluster of platelets at the apex is called the apical pore complex (APC). Those in the sulcus are termed sulcals. Often there is a midventral plate on the epitheca that stretches from the apex to the girdle. Traditionally this is termed the first apical plate. The plates are numbered around to the cell's left (Fig. 15.1), continuing around the dorsal side to the end of the series,

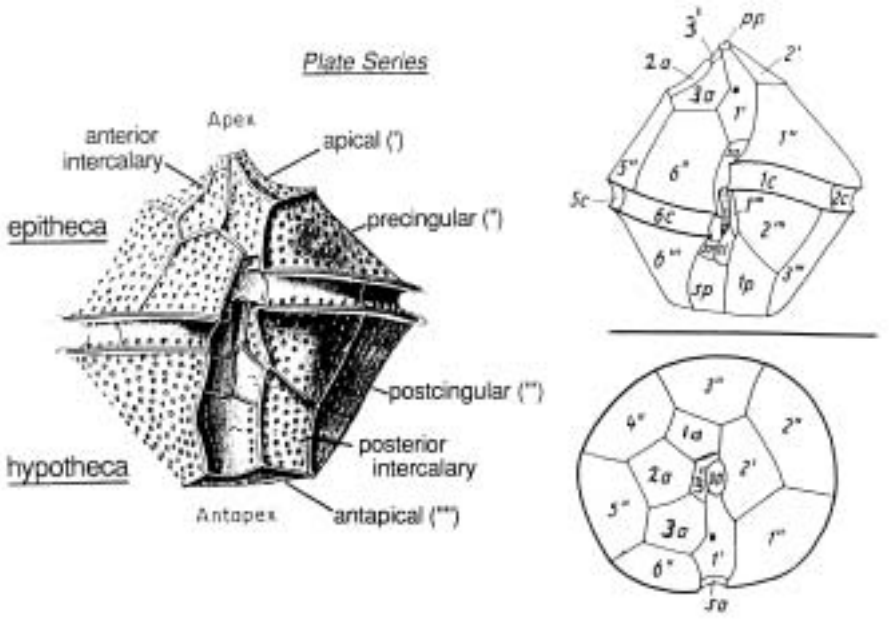


Figure 15.1
Dinoflagellate plate tabulation as exemplified by the gonyaulacoid *Lingulodinium polyedrum*.
Plate formula Po, 3′, 3a, 6″, 6c, 7s, 6‴, 1p, 1⁗.

starting from the plate nearest the midventral region. The apicals contact the APC, the precingulars contact the anterior edge of the girdle (cingulum). The cingulars are in the girdle (another name for which is cingulum). The postcingulars contact the posterior edge of the girdle and the antapicals lie at the antapex.

All the above features are morphological. Such morphologically defined species are termed morphospecies (Taylor, 1992, 1993). In general there is agreement between morphological and other cell features but recognition is increasing of genetic variability within morphospecies. For example, toxicity cannot always be assumed for a particular morphospecies: in some all strains tested have been found to be toxic, such as *Alexandrium catenella* and possibly some *Dinophysis* spp. In others non-toxic strains are known, such as *Alexandrium tamarense* and *Gambierdiscus toxicus*, or toxin production may be environmentally inducible. On the other hand, cells that are morphologically *A. catenella* from Japan, Chile and the North American west coast not only look alike, but even make the same suite of toxins.

Life-cycle features are also very important and all stages, when known, play a part in recognition of species. In this publication the use of cysts (dormant benthic stages) in identification is dealt with in Chapter 20.

15.5 Basic types of dinoflagellate involved in harmful blooms

Dinoflagellates show a great range of forms but most of this diversity can be resolved to five basic types (Taylor, 1980) and the harmful species are dealt with under these categories below.

15.5.1 Gymnodinioids and noctilucoids

The taxonomy of the unarmoured dinoflagellates is still poorly known and not very robust. Several genera of unarmoured dinoflagellates, such as *Amphidinium*, *Gymnodinium* and *Gyrodinium*, comprise photo-, hetero- and mixotrophic species, and different types of chloroplast in some genera, which is highly unusual from a taxonomic point of view (Larsen and Sournia, 1991). Definition of taxa is almost invariably based on classical morphological characters, whereas fine structural features which are now widely used to define taxa in other groups of flagellates, as well as molecular data (Daugbjerg *et al.*, 2000) are only beginning to show their impact. Fine structural and molecular studies on species of the well-established genus *Gymnodinium*, albeit still few in number, indicate its polyphyletic nature, and hence we can expect this taxon to be divided into a number of genera in the future. The flagellar apparatus of *Gymnodinium sanguineum* Hirasaka (now transferred to the new genus *Akashiwo* G. Hansen and Moestrup), thus shows greater similarity to *Polykrikos kofoidii* Chatton than any other species of *Gymnodinium* (Roberts, 1991). Any taxonomic revisions of this group need to be based on a combination of morphological, fine structural, molecular and pigment, lipid and toxin biomarker information, and will require a re-examination of the type species of the different genera of unarmoured dinoflagellates. It should not be expected that all unarmoured dinoflagellate cells in a given sample are identifiable. Some species are quite robust and persist for many minutes when observed under the microscope, while the most delicate species deteriorate after a few minutes of observation. Several species of unarmoured

dinoflagellates are undoubtedly based on preserved material (Schiller, 1933) and many poorly described species probably can never be re-identified.

15.5.1.1 *Amphidinium* Claparède et Lachmann

The girdle is strongly dislocated towards the anterior end of the cell, so the episome is small, often tongue-shaped or appearing as a small 'cap' on the top of the cell. However, considerable morphological variation is seen in the genus, and in some species the epicone and the hypocone are almost equal in size. Thus, the genus is not clearly delimited from *Gymnodinium* and assignment of species is often arbitrary. The type species of *Amphidinium, A. operculatum* Claparède et Lachmann has a finger-like epicone, and constitutes together with *Amphidinium carterae* Hulburt and *A. rhynchocephalum* Anissimowa what may currently be conceived as the true *Amphidinium*. Molecular data suggests that this group of species is not closely related to *Gymnodinium* (Daugbjerg *et al.*, 2000). *Amphidinium* presently comprises about 100 photo- or heterotrophic species, but many of them will need to be re-examined to determine their generic affiliation. The genus is found mainly in salt- or brackish water habitats, and many species are benthic.

Amphidinium carterae Hulburt (Fig. 15.2)

More or less oval cells, flattened dorso-ventrally. The episome is small, crescent- or tongue-shaped, deflected to the left, describing a descending spiral which is displaced two to three girdle widths. One widely branched and usually peripheral chloroplast with a large central pyrenoid. The nucleus is crescent-shaped and located in the hyposome. Length 12–18 µm, width 8–10 µm.

Amphidinium carterae belongs to a group of species that includes *A. operculatum* Claparède et Lachmann, which is the type species of the genus, and *A. klebsii* Kofoid et Swezy. *Amphidinium carterae* and *A. operculatum (*as *A. klebsii*) are the most often reported species of this complex, but cannot always be clearly distinguished. According to Taylor (1971), they can be distinguished by the morphology of the chloroplast. The size of the cells should also be considered; *A. carterae* appears to be smaller (Hulburt (1957) indicated a length of 12–15 µm) than *A. operculatum* (Kofoid and Swezy (1921) reported 46 µm for *A. klebsii*). *A. carterae* can produce hemolytic compounds (Tindall and Morton, 1998).

Distribution: This species is presumably widely distributed. It has been reported from both temperate and tropical areas. However, due to persisting taxonomic problems, many reports are difficult to assess.

Amphidinium operculatum Claparède et Lachmann (Fig. 15.3)

Synonym: *A. klebsii* Kofoid et Swezy

This species is similar to *A. carterae* in having more or less oval cells with a small tongue-shaped, leftward-deflected episome. It contains numerous long, slender chloroplasts attached to a large pyrenoid located in the central part of the hyposome. Length 20–46 µm, width 14–30 µm.

This species may be difficult to distinguish from *A. carterae*, but can be identified by the larger size and by the numerous slender chloroplasts deployed around the large pyrenoid (Taylor, 1971), see also comments on *A. carterae*.). *A. operculatum (*as *A. klebsii*) can produce hemolytic compounds (Tindall and Morton, 1988).

Distribution: Amphidinium operculatum has on several occasions been reported from the same sites and localities as *A. carterae*. It is not at present possible to establish the precise distribution of the two species.

15.5.1.2 *Cochlodinium* Schütt

Cochlodinium is characterized by a descending girdle that performs 1.5 to 4 turns around the cell. The genus comprises about 40 species, most of which are heterotrophic (Larsen and Sournia, 1991). Many are poorly known and in need of re-examination.

Cochlodinium catenatum Okamura, *C. citron* Kofoid et Swezy, as well as several unidentified species, have been associated with harmful events, but only *C. polykrikoides* is included here as this species causes the most serious problems.

Cochlodinium polykrikoides Margalef (Fig. 15.4)

Synonyms: *C. heterolobatum* Silva, C. type ′78 Yatsushiro

The cells often form short chains rarely consisting of more than eight cells. The individual cells are more or less oval, only slightly flattened and with a girdle making 1.8–1.9 turns around the cell, notched at the antapex. An apical groove is present. A red stigma is located on the dorsal side of the episome. There are several band-shaped chloroplasts. The nucleus is located in the episome. Length 30–40 µm, width 20–30 µm.

Distribution: Cochlodinium polykrikoides has been reported from the American east coast, Japan where it is widely distributed and often forms harmful blooms, Korea and Puerto Rico.

Comments: This species may be toxic to juvenile fish, but the toxic principles are unknown.

15.5.1.3 *Gymnodinium* Stein emend. G. Hansen and Moestrup

Unarmoured dinoflagellates with the cingulum located in the equatorial region of the cell, and with a displacement less than 1/5 of the cell length, are traditionally referred to *Gymnodinium* (Kofoid and Swezy, 1921). According to the emended description of the genus, true *Gymnodinium* are characterized by certain ultrastructural features and by a horseshoe-shaped apical groove running in an anticlockwise direction (Daugbjerg *et al.*, 2000). The type species of *Gymnodinium*, the freshwater dinoflagellate *G. fuscum*, exhibits some profound ultrastructural differences from marine counterparts, and it is likely that in future many marine gymnodinioids will need to be transferred to new genera.

As redefined, the genus *Gyrodinium* (type species *G. spirale* (Bergh) Kofoid and Swezy) comprises exclusively naked, heterotrophic dinoflagellates possessing an amphiesma with longitudinal striations. The cingulum displacement ranges from one to more cingulum widths. The apical groove is an elliptical structure, perpendicular to the longitudinal axis of the cell. No harmful species are known in the latter genus.

Gymnodinium catenatum Graham (Fig. 15.5)

The cells form chains of usually 4, 8 or 16 cells, occasionally longer. Unfavourable conditions may cause the chains to break up into single cells, and non-chain-forming clones possibly also exist. The girdle describes a descending spiral, which is displaced up to 1/5 of the cell length. The sulcus extends from the antapex to the apex, which is also surrounded by a semicircular apical ring. The cells contain numerous chloroplasts with conspicuous pyrenoids. The nucleus is located in the central part of the cell. The reported size ranges are for solitary cells: 34–65 µm long and 27–43 µm wide; for chain-forming cells 23–60 µm long and 27–43 µm wide, with the terminal cells being about the size of solitary cells.

Gymnodinium catenatum is the only unarmoured dinoflagellate known to produce PSP toxins. It is distinguished from other species of *Gymnodinium* by its chain-forming habit and may be confused only with the non-toxic *Gymnodinium impudicum* (Fraga and Bravo) G. Hansen and Moestrup. *Gymnodinium impudicum* is distinguished by the smaller size (about 25 µm long) and the apical groove, which is an extension of the sulcus (see also Hallegraeff and Fraga, 1998). Single cells of *Gymnodinium catenatum* are best identified by the large pyrenoids and the conspicuous sulcus. The life cycle of *G. catenatum* was described by Blackburn *et al.* (1989), who found that sexual reproduction may be induced by nutrient deficiency, and that the gametes are indistinguishable from vegetative cells (hologamy) and heterothallic in nature. The resulting resting cyst (hypnozygote) is round (42–52 µm diameter) and brown with microscopic reticulations. Two other gymnodinioid species produce similar, albeit smaller, microreticulate resting cysts, *Gymnodinium nolleri* Ellegaard et Moestrup and *Gymnodinium microreticulatum* Bolch et Hallegraeff. Neither of these species has been associated with shellfish toxicity.

Distribution: This species was first reported from California (USA) and has subsequently been reported from several places including Argentina, Australia (Tasmania), New Zealand, Japan, Korea, Mexico, Philippines, Portugal, Singapore, Spain and Venezuela. According to Hallegraeff and Bolch (1992), dinoflagellate cysts may travel considerable distances in ships' ballast water, and *G. catenatum* could have been introduced into Australian waters in this way.

Gymnodinium pulchellum Larsen (Fig. 15.6)

Synonyms: *Gymnodinium type '*84K (Onoue et al., 1985; Fukuyo et al., 1990; Hallegraeff, 1991); *Gymnodinium* sp. 1 (Takayama, 1985)

The cells are broadly oval and only slightly flattened. The girdle is pre-median and describes a descending spiral which is displaced 1–1.5 girdle widths. The sulcus intrudes for a short distance on to the episome, which also has a sigmoid apical groove. It has several irregular, chloroplasts with pyrenoids. The nucleus is located in the left part of the cell. Length 16–25 µm, width 11–16 µm. *Gymnodinium pulchellum* is distinguished from *Karenia mikimotoi* by the sigmoid apical groove. The precise generic affiliation of this species is currently under revision.

Distribution: Gymnodinium pulchellum was described from the Melbourne area, Australia (Larsen, 1994). It has been reported also from Tasmanian waters (Hallegraeff, 1991) and from Japan (Onoue *et al.*, 1985, as *G.* type '84K; Takayama, 1985 as *G.* sp. 1), Florida, Italy and New Zealand.

15.5.1.4 *Karenia* G. Hansen et Moestrup

This genus was created to accommodate gymnodinioid species with a straight apical groove and with fucoxanthin derivatives as accessory photosynthetic pigments (Riley and Wilson, 1967; Jeffrey *et al.*, 1975; Tangen and Bjornland, 1981; Rowan, 1989). It comprises the well-known toxic species *K. brevis* (synonym *Gymnodinium brevis*), *K. mikimotoi* and the recently described species *K. brevisulcata.*

Karenia brevis (Davis) G. Hansen et Moestrup (Fig. 15.7)

Synonyms: *Gymnodinium breve* Davis, *Ptychodiscus brevis* (Davis) Steidinger

The cells appear almost square in outline, but with a prominent apical process; strongly flattened dorso-ventrally. The girdle is not (or only slightly) displaced and then describes a descending spiral; three vertical ridges are present near the distal end of the girdle. The sulcus intrudes on to the episome. An apical groove extends

from near the sulcal extension on the episome across the apical process and on to the dorsal side of the cell. There are numerous chloroplasts with pyrenoids.

Comments: Reports of *K. brevis*-like species outside the Gulf of Mexico and the Western Atlantic region may represent different, closely related species (Steidinger *et al.*, 1989), including *K. papilionacea, K. bicuneiformis* and *K. selliformis* to be described from New Zealand (Haywood and Steidinger, in press). The toxins are responsible for neurotoxic shellfish poisoning (NSP) which may be harmful to both fish and mammals, while aerosolization of the toxins may be responsible for asthma-like symptoms. NSP has been restricted so far to the south-eastern USA, particularly the Gulf of Mexico, and New Zealand.

Distribution: Karenia brevis occurs regularly in the Gulf of Mexico, but *K. brevis* or *K. brevis*-like species have also been reported from the West Atlantic, Spain, Greece, Japan, Australia and New Zealand.

Karenia brevisulcata (Chang) G. Hansen and Moestrup (Fig. 15.9)
Basionym: *Gymnodinium brevisulcata* Chang

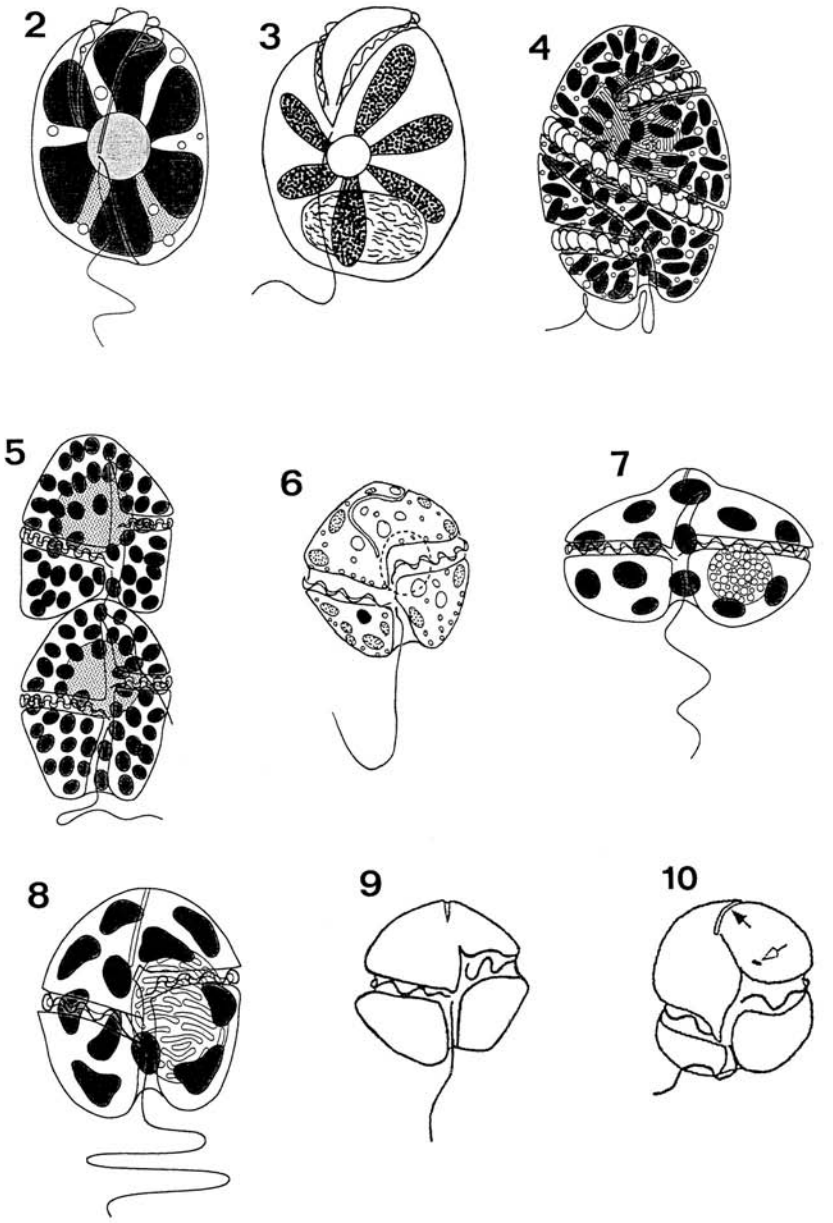
This is the smallest species in the *mikimotoi* group, 18–37 µm long; 18–22 µm wide, slightly flattened, with a round nucleus, either located in the left hypocone, or stretching horizontally from the left to right hypocone. Cingulum displacement is 11–27% of body length. It has a straight, very short apical groove, located to the right of the sulcal axis, and extending 1/3 to 1/2 on the ventral side and 1/3 down the dorsal side of the epicone. A small triangular sulcus extension occurs into the epicone.

This species caused extensive fish kills in the summer of 1998 in Wellington harbour, New Zealand (Chang, 1999).

Karenia mikimotoi (Miyake et Kominami ex Oda) G. Hansen and Moestrup (Fig. 15.8)
Synonyms*: Gymnodinium mikimotoi* Miyake et Kominami ex Oda, *Gymnodinium nagasakiense* Takayama et Adachi*, Gymnodinium* sp. 1 (Adachi, 1972)*, Gymnodinium* type '65 (Iizuka and Irie, 1969)*, Gyrodinium aureolum* Hulburt, sensu Braarud and Heimdal (1970)

The cell outline is variable, ovate to almost round, flattened dorso-ventrally. The girdle is wide and describes a descending spiral which is displaced about 1/5 of the cell length. The sulcus continues for a short distance on to the episome, where an apical groove extends in a straight line from near the sulcal intrusion across the apex and a short distance down on the dorsal side of the cell. There are several more or less oval chloroplasts, each with a conspicuous pyrenoid. The nucleus is located in the left side of the hypocone. Length 24–40 µm, width 20–32 µm.

The morphological similarity between the Pacific *K. mikimotoi* and the European *Gyrodinium aureolum* has been noted by several authors (Taylor, 1985; Partensky *et al.*, 1988; Fukuyo *et al.*, 1990; Hallegraeff, 1991). A detailed study of this problem was carried out by Hansen *et al.* (2000), who concluded that all isolates of *G. aureolum* or *G. nagasakiense* from Europe are conspecific with Japanese strains of *K. mikimotoi*, and that *Gyrodinium aureolum* is a different taxon which in turn was transferred to *Gymnodinium*. The latter differs from *K. mikimotoi* in several details such as position of the nucleus, dorso-ventral flattening of the cell, number of chloroplasts and pyrenoids, and having a horseshoe-shaped apical groove (as *Gyrodinium* sp. 1 of Bolch and Hallegraeff, 1990). *Karenia mikimotoi* produces both hemolytic and ichthyotoxins, and has caused damage to fish farms in northern Europe, Japan and New Zealand.



Figures 15.2–10
Gymnodinioid dinoflagellates. 2, *Amphidinium carterae*;
3, *A. operculatum (= klebsii)*; 4, *Cochlodinium polykrikoides*;
5, *G. catenatum*; 6, *G. pulchellum*; 7, *Karenia brevis*;
8, *K. mikimotoi*; 9, *K. brevisulcata*; 10, *Karlodinium micrum*.
Sources: 2, 4, 5, 7, 9, Takayama; 3, after Taylor (1971); 6, Matsuoka;
8, Chang; 10, Larsen.

Distribution: K. mikimotoi is widely distributed and has formed blooms in Australia, Denmark, Ireland, Japan, Korea, Norway and Scotland.

Comments: A number of other *Karenia* species have been associated with aquaculture fish kills, such as *Karenia digitata* Yang, Takayama, Matsuoka and Hodgkiss in Japan and Hong Kong (Yang *et al.*, 2000). This species is named for a diagnostic short finger-like sulcus extension into the epicone. In *Karenia longicanalis* Yang, Hodgkiss et Hansen the apical groove is straight, located to the right of the sulcal axis, and extending 2/3 down the dorsal side of the epicone (Yang et al, 2001).

15.5.1.5 *Karlodinium* J. Larsen

This genus is characterized principally by ultrastructural features, viz. an amphiesma with arrays of plug-like structures in a hexagonal configuration. In addition, the cells have chloroplasts with internal, lenticular pyrenoids and fucoxanthin or fucoxanthin derivatives as accessory pigments. The apical groove is straight.

Karlodinium micrum (Leadbeater and Dodge) J. Larsen (Fig. 15.10)
Synonyms*: Woloszynskia micra* Leadbeater and Dodge, *Gymnodinium micrum* (Leadbeater et Dodge) Loeblich III, *non Gymnodinium galatheanum* Braarud *sensu* Kite and Dodge, *Gymnodinium galatheanum* (Braarud) Taylor

The cell shape is oval to round. The girdle is deeply incised, describing a descending spiral which is displaced almost 1/5 of the cell length. The sulcus is strongly deflected in the inter-cingular region and extends on to the episome. The cell usually contains two chloroplasts, one in the epicone and one in the hypocone, which are visible by light microscopy. The nucleus is located in the central part of the cell. Length 9–17 µm, width 8–14 µm. The gross morphology of *K. micrum* is shared by several small species of unarmoured dinoflagellates including *G. corsicum* Paulmier, Billard and Nezan. This species is toxic to mussels and juvenile cod (*Gadus morhua*).

Distribution: This species has been reported from the North and South Atlantic, as well as Australia. It is presumably widespread, but easily overlooked because of its small size.

Comments: The fish killer *Karlodinium veneficum* (Ballantine) J. Larsen (= *Gymnodinium veneficum* Ballantine) is not easily distinguished from *K. micrum*, but it usually has four chloroplasts and a less-pronounced sulcal deflection in the inter-cingular region as compared to *K. micrum*. It was first described from a culture isolated from the English Channel (Ballantine, 1956), but is presumably widely unreported due to its small size. The similarly sized *K. vitiligo* (Ballantine) J. Larsen is non-toxic.

15.5.1.6 *Noctiluca scintillans (*Macartney*) Kofoid* (Fig. 15.11)

Synonym: *N. miliaris* Suriray

The cells are large and more or less balloon-shaped, with a striated tentacle. *N. scintillans* is phagotrophic and the cells are usually colourless. In certain tropical area it may attain a green colour because of the presence of endosymbiotic flagellates (Sweeney, 1976). Diameter 200–2,000 µm.

Blooms of *N. scintillans* may cause a strong red discoloration of the water. It may be bioluminescent except in parts of the northern and eastern Pacific Ocean. It accumulates large amounts of ammonia, which may be toxic to fish, in the vacuole.

Distribution: N. scintillans is widely distributed and it has formed blooms in temperate, subtropical and tropical waters.

15.5.2 Peridinoids

This thecate group is one of two in which the plates are arranged in the classic five-horizontal-plate series, the other being gonyaulacoids (below, see Fensome *et al.*, 1993, for a full discussion of this). Many are non-photosynthetic although the harmful species below is photosynthetic.

15.5.2.1 *Peridinium, 'Glenodinium'*

The older literature is confusing in that all marine *Protoperidinium* species were included in the genus *Peridinium*, but the latter is now restricted to species with five or six cingular plates (instead of three plus a small transitional in *Protoperidinium)*. This action also resulted in the marine species of the latter genus all being non-photosynthetic whereas it appears that the *Peridinium* species are all photosynthetic. *Glenodinium* has been used variously in the past but, if one uses the type species as an indication of its character, the species below is clearly not a *Glenodinium*.

15.5.2.2 *Peridinium polonicum* Woloszynska (Fig. 15.12)

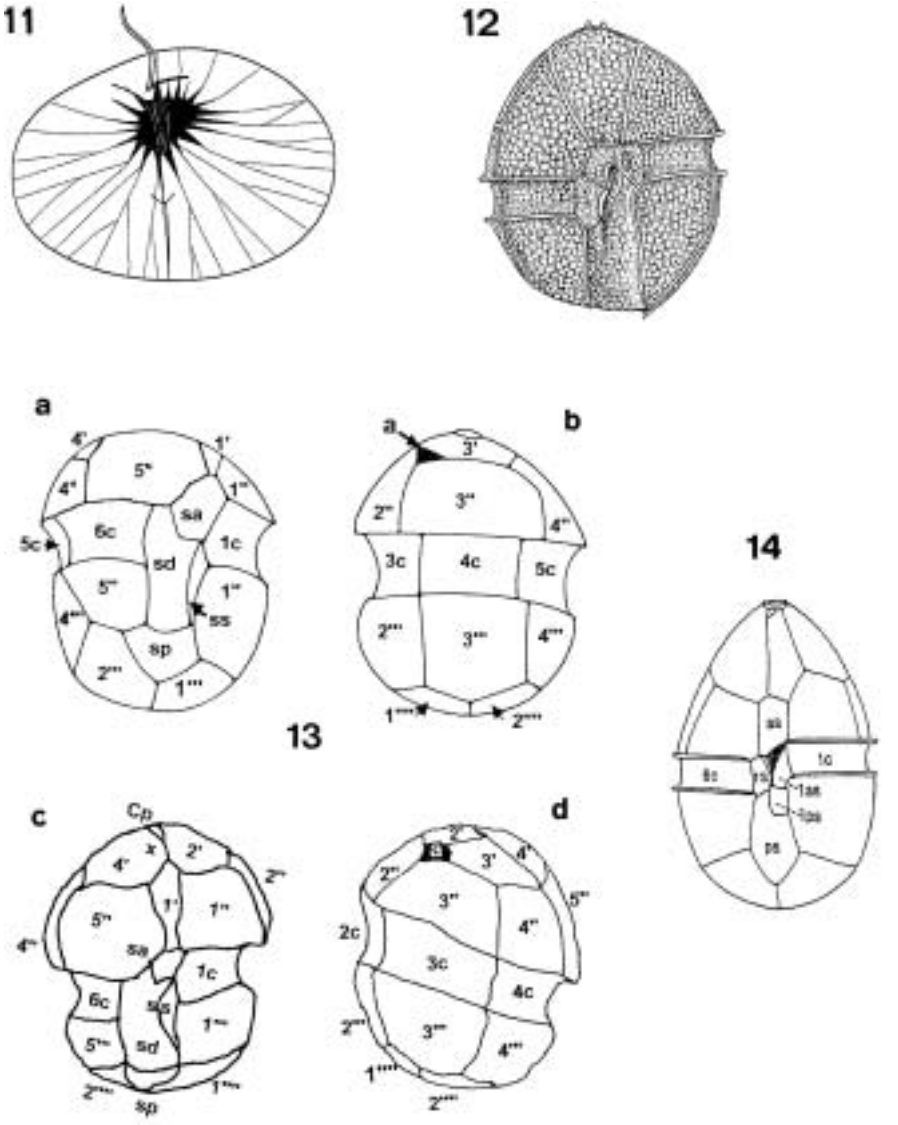
Synonyms: *Peridiniopsis polonicum* (Woloszynska) Bourrelly, *Glenodinium gymnodinium* Penard?

Ovoid cells, somewhat flattened dorso-ventrally. The girdle is slightly indented, lacking lists, median with a left-handed displacement of one girdle width. The sulcus is straight, slightly wider than the girdle, only indenting the posterior profile very slightly. The first apical plate has four main sides ('ortho') and two small, unequal anterior intercalaries (sometimes only one and thence put in the genus *Peridiniopsis*). The two antapicals are also unequal, the second being bigger than the first. Length 34–43 µm, transdiameter 28–38 µm. The only freshwater dinoflagellate to be positively linked to harmful blooms, being linked to fish kills.

Distribution: First described from Europe; the harmful effects of this species have occurred in Japan.

15.5.2.3. *Pfiesteria piscicida* Steidinger et Burkholder (Figs. 15.13A, B)

The ichthyotoxic 'phantom' dinoflagellate *Pfiesteria piscicida* was first recognized in North Carolina in 1988 (Burkholder *et al.*, 1992). Its ephemeral presence (cysts germinate in the presence of live fish, and encyst again after fish death) may explain many mysterious fish kills along the south-east coast of the USA. The organism was described as a new species and genus within the order Dinamoebales (Steidinger *et al.*, 1996). This dinoflagellate has a reported multiphasic life cycle with polymorphic and multiphasic bi- and triflagellated, amoeboid, and non-motile cyst stages with transitional forms between all stages. All stages are unicellular. Bi- and triflagellate stages are typically planktonic and ephemeral and resemble gymnodinioid forms but are actually small cryptic peridinioids with a plate formula of Po, cp, X, 4′, 1a, 5″, 6c, 4s, 5‴ and 2⁗. Biflagellate stages are of two size classes, 5–8 µm and 10–18 µm, both regarded as haploid. Triflagellate stages, 25–60 µm, with one transverse and two longitudinal flagella, are regarded as planozygotes. Amoeboid stages vary in size (5–250 µm), shape and type of podia. Filose and lobose amoeboid stages are thecate with a honeycomb-like surface structure of repeated coalesced units similar to *Heterocapsa* or *Katodinium* organic scales. Cyst stages, 25–33 µm, range from spherical testate forms with a honeycomb surface pattern to forms resembling scaled chrysophytes. The cells are heterotrophic and mixotrophic, capable of phagocytosis, and with cleptochloroplasts present.



Figures 15.11–14
11, *Noctiluca scintillans*;
12, *Peridinium polonicum*;
13A, 13B, *Pfiesteria piscicida*;
13C, 13D, *Pfiesteria shumwayae*;
14, *Heterocapsa circularisquama*.
Sources: 11, Takayama;
12, Inoue; 13, Burkholder and Glasgow;
14, Horiguchi.

15.5.2.4. *Pfiesteria shumwayae* Glasgow et Burkholder (Fig. 15.13C, D)

A second toxic species within the family Pfiesteriaceae was described from the New River Estuary, USA (Glasgow *et al.*, 2001). It can be distinguished morphologically by having 6′ plates and a four-sided 1a plate (as opposed to a triangular 1a plate in *P. piscicida*), but in practice DNA probes are needed for species discrimination. Toxic strains of the two *Pfiesteria* species have overlapping distributions in the mid-Atlantic and south-eastern USA and in Scandinavia, with both *P. shumwayae* and *P. piscicida* also verified from New Zealand and Australia.

15.5.2.5 *Heterocapsa circularisquama* Horiguchi (Fig. 15.14)

Small photosynthetic dinoflagellate cells, 20–29 µm long, 14–20 µm wide, with conical epitheca and hemispherical hypotheca and thecal plate arrangement Po, cp, 5′, 3a, 7″, 6c, 5s, 5‴, 2⁗. The species is named for the diagnostic body scales with six radiating ridges on a circular basal plate, but which are visible only by transmission electron microscopy. All other known dinoflagellate body scales, such as from *Heterocapsa (Katodinium) rotundata* and *Heterocapsa triquetra*, are triangular in outline. This species has caused mass mortality of bivalves such as oysters, short-necked clams and pearl oysters in Japan (Horiguchi, 1995). Strains of *H. rotundata* have also been demonstrated to be toxic to *Artemia* (LeRoi, unpublished).

This small dinoflagellate was first recognized in Japan in 1988, and has since spread to many other areas, particularly of western Japan. It is unclear whether the species is indigenous to Japan or perhaps was introduced from tropical regions through transfer of shellfish stock or advection in prevailing northward currents.

15.5.3 Gonyaulacoids

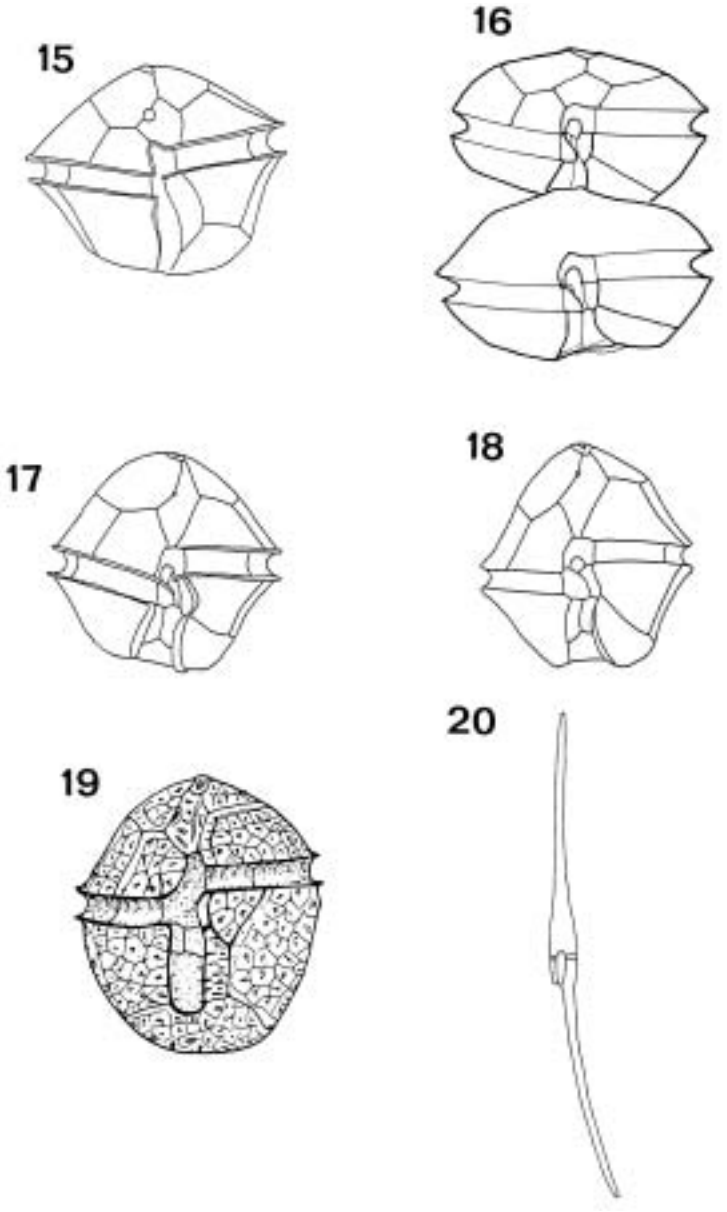
These are similar to the peridinioids but differ in the symmetry of their thecal plates (see Taylor, 1980, 1987; Evitt, 1985; Fensome *et al.*, 1993). They are predominantly photosynthetic and the majority of toxic species belong to this assemblage.

An important feature used in generic distinctions is the shape and relationships of the homologue of the traditional ‘rhomb’ plate (Kofoidean first apical). Within gonyaulacoids it may extend all the way to the APC, in which it is a typical first apical plate. If not to varying degrees, in which it would then be, strictly speaking, a first postcingular plate. Evitt (1985) recognized three states, named as insert, metasert and exsert.

15.5.3.1 *Alexandrium, Goniodoma* (Figs. 15.15–20, 15.21)

Synonyms: *Gessnerium* Halim, *Protogonyaulax* Taylor

This gonyaulacoid genus has the most species producing paralytic shellfish poisons although not all members are toxic. For a number of years *Alexandrium* and *Protogonyaulax* were held separate (*Gessnerium* being an erroneously optically inverted *Alexandrium*), with those species in which the first apical plate contacted the APC (‘insert’). The majority of toxic species, assigned to *Protogonyaulax*, and those in which they did not contact one another (‘metasert’) assigned to *Alexandrium* even though it was evident that they were closely related (Taylor, 1979, 1985). Variability of this condition in *A. minutum*, the type species of *Alexandrium*, has led to an abandonment of this position. The genus has been reviewed in outline by Balech (1985)



Figures 15.15–20
Different *Alexandrium* morphotypes. 15, *A. hiranoi*, with cingulum above the middle; 16, chain-forming *A. monilatum*; 17, round cell type of *A. catenella*, *A. minutum*, *A. ostenfeldii*, *A. tamarense*, with cingulum in the middle; 18, angular cell type of *A. acatenella*, *A. angustitabulatum*, *A. cohorticula*, *A. tamiyavanichi*, with cingulum in the middle; 19, *Protoceratium reticulatum*; 20, *Ceratium fusus*. *Sources:* 15, Takayama; 16, Balech; 19, Dodge; 17–18, 20, Inoue.

and in detail by Balech (1995). A number of the species recognized in these reviews are considered to be infraspecific variants here and elsewhere (Cembella and Taylor, 1986; Taylor, 1992) as the result of culture-based studies.

Generic characterization: thecate gonyaulacoid cells with the Kofoidean plate formula: P, 4′, 6″, 6c, 9–11s, 5‴, 1p, 1⁗ (or 2⁗ if the 1p is interpreted as in Balech and Tangen, 1985). The girdle has a left-handed displacement of one girdle width. Horns and spines are absent. Girdle margins have very low ridges. The APC has a triangular outer plate (Po) and a comma-shaped inner opening. An accessory pore may be present on Po and on the posterior sulcal when the cells are in pairs or chains. The first apical plate (1u in the Taylor-Evitt system), or its homologue when not in contact with the APC, is usually five-sided and insert or metasert. Plate surfaces usually lack ornamentation other than pores. The resting cyst is usually smooth and ovoid to round and has a chasmic (slit-like) archeopyle.

The descriptions here are limited to those known or strongly suspected to be toxic. However, as so many species of this genus are toxic and there is variability in toxicity in some of them, a bloom of any member of this genus should be viewed with concern for possible PSP in local shellfish (for other species such as *A. andersoni, A. balechii, A. compressum, A. fraterculus, A. insuetum, A. pseudogonyaulax, A. taylori,* etc., see Balech, 1995).

15.5.3.2 *Alexandrium acatenella* (Whedon and Kofoid) Balech (Fig. 15.21)

Synonyms: *Gonyaulax acatenella* Whedon and Kofoid, *Protogonyaulax acatenella* (Whedon and Kofoid) Taylor

Cells single, medium-sized, longer than wide with a slightly angular outline when seen in ventral view. The epitheca is longer than the hypotheca (roughly the same length as the latter plus the girdle). In some individuals the APC may project slightly. It is narrowly rectangular in shape. The suture between the second postcingular and the conventional posterior intercalary may be ridge-like in British Columbia. The surface is clearly porulated. A ventral pore is present. Balech reports a pore on the posterior sulcal plate in Japanese material. Length 35–51 µm (most near 40 µm), transdiameter 26–35 µm. This species is only dubiously separated from *A. tamarense* by the proportions of the epitheca to hypotheca. Balech notes that it has a wider posterior sulcal plate. A population closely resembling the original description was responsible for human illness and a fatality in British Columbia in 1965 (Prakash and Taylor, 1966).

Distribution: West coast of North America, Japan, Argentina, possibly Chile.

15.5.3.3 *Alexandrium angustitabulatum* Taylor in Balech (Fig. 15.21)

Another small species resembling *A. minutum*. The ventral view is ovoid and can appear to have a symmetrical posterior indentation if slightly tilted. The epitheca is slightly larger than the hypotheca. The APC is almost rectangular. The first apical plate usually contacts the APC. It lacks a ventral pore. Its two longest margins are parallel and the suture with the sixth precingular plate is short. The sixth precingular is narrow. The anterior sulcal is subequal in length and breadth or slightly wider. Length 17–25 µm, transdiameter 14–24 µm. A culture of this species has been shown to produce toxins. The shape of the first apical plate and lack of a ventral pore distinguish the species from *A. minutum*.

Distribution: This species was isolated from mixed material from Whangarei Harbour, New Zealand' in 1983.

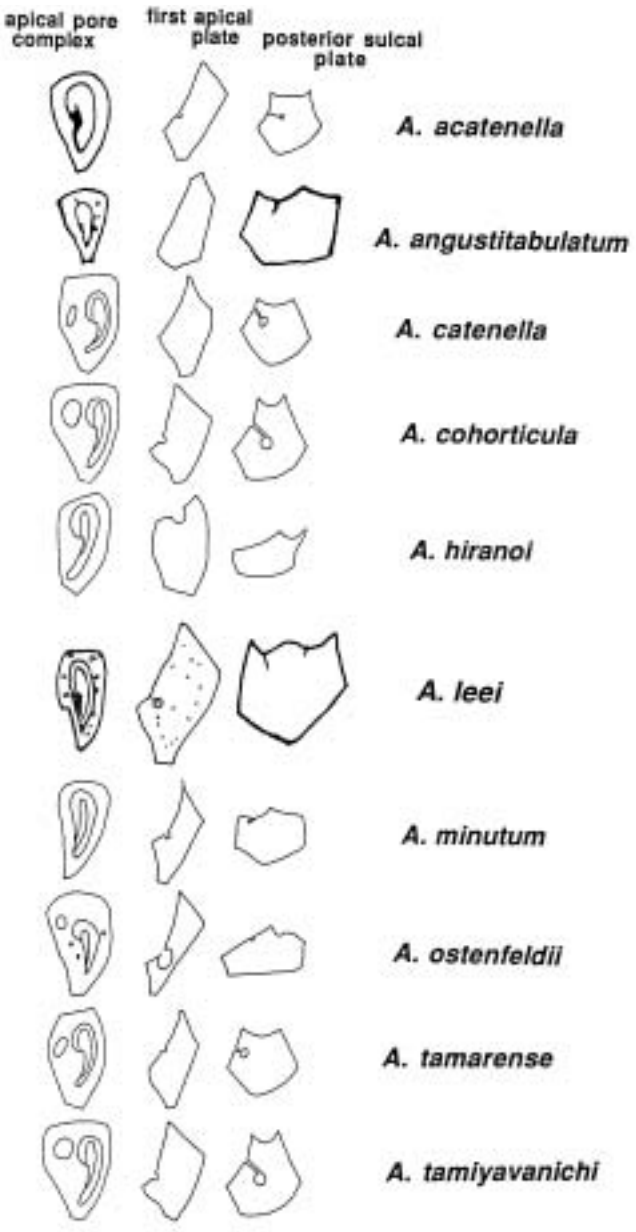


Figure 15.21
Shapes of the three key diagnostic plates (apical pore complex, first apical plate, posterior sulcal plate) of (in alphabetical order) *Alexandrium acatenella*, *A. angustitabulatum*, *A. catenella*, *A. cohorticula*, *A. hiranoi*, *A. leei*, *A. minutum*, *A. ostenfeldii*, *A. tamarense* and *A. tamiyavanichi*. *Sources:* Fukuyo and H. Inoue; *A. acatenella*, *A. angustitabulatum*, *A. leei*, after Balech (1995).

15.5.3.4 *Alexandrium catenella* (Whedon and Kofoid) Balech (Fig. 15.21)

Synonyms: *Gonyaulax catenella* Whedon and Kofoid, *Protogonyaulax catenella* (Whedon and Kofoid) Taylor

A medium-sized species. Typically in field samples this species forms long chains of cells distinctly wider than long. In culture and some Japanese populations the cells may be isodiametric. The surface is lightly porulated. The epitheca and hypotheca are equal in length. The first apical plate usually has strong contact with the APC. A ventral pore is consistently absent. The APC is broad and subtriangular. In chains both anterior and posterior attachment pores are present. The posterior sulcal plate is wide, almost five-sided, the attachment pore near the right margin. Length 20–40 µm, transdiameter 18–37 µm. This was the first species to be linked to PSP. It seems to be consistently toxic. The cell shape is distinctive in chains and distinguishes it from other chain formers except *A. tamiyavanichi* Balech. The latter, a warm-water species, has a distinct anterior projection to its anterior sulcal plate and a posterior sulcal which is not as wide. Chain length varies and the chains can break when sampled. In culture single cells can occur.

Distribution: This species is widely distributed in cold temperate waters, including the west coast of North America from California to Alaska, Japan, Chile, Argentina, New Zealand and Australia.

15.5.3.5 *Alexandrium cohorticula* (Balech) Balech (Fig. 15.21)

Synonyms: *Gonyaulax cohorticula* Balech, *Protogonyaulax cohorticula* (Balech) Taylor

A larger, chain-forming species. The cells are rounded, slightly longer than wide. The surface is finely, densely porulated. The epitheca lacks angularity at the 'shoulders' and is slightly longer than the hyoptheca. The first apical plate contacts the APC and its right upper margin is concave with a ventral pore present. The APC is broadly triangular with a larger anterior connecting pore. The anterior sulcal plate has a distinct squarish to trapezoid anterior projection demarcated by a ridge on the plate. The margins of the posterior sulcal plate may be flange-like. The toxicity of this species is now in question as the culture from the Gulf of Thailand that was shown to be toxic is now referred to by Balech as a recently described species, *A. tamiyavanichi*. The latter is very similar (see description below).

Distribution: A warm-water species recorded from the Gulfs of Mexico and California (Balech) and Phuket, Thailand (Taylor, unpublished).

15.5.3.6 *Alexandrium hiranoi* Kita and Fukuyo (Fig. 15.21)

Cells are ovoid to round in ventral view, slightly longer than wide or subequal. The size range is considerable. The APC is narrowly rectangular with a hook-like apical pore. A small ventral pore is present on the upper right boundary of 1u. The girdle is median, displaced one girdle width. The sulcus is shallow, with the posterior sulcal homologue (Z of T-E) not depressed and thus resembling an antapical. Thecal pores sparse. An unusual, distinctive feature is its mode of division in a non-motile, cyst-like state. A smooth-walled resting cyst is also present. Length 18–75 µm (most 40 µm), transdiameter 18–75 µm (most 35–40 µm). This species is closest to *A. pseudogonyaulax* but differs from it in that the latter is different in shape (flattened, wider than long). Other differences noted by the original authors are either the consequence of this broader shape (wider first apical homologue, 1u) or the result of

more detailed examination. They may well prove to be conspecific but a difference in the resting cyst ornamentation (reticulate in *A. pseudogonyaulax*) may also serve to separate them.

Distribution: Species known from tide pools in Japan and British Columbia coastal waters.

15.5.3.7 *Alexandrium leei* Balech 1985 (Fig. 15.21)

A relatively large, rounded, non-chain-forming species. The antapex is notably asymmetrical with a rounded lobe on the lower left side, so that the left side projects antapically much more than the right side. The APC is narrow and points toward the sulcus without any large accessory pore. The first apical plate is relatively wide, its upper right edge curving smoothly and a small ventral pore is located significantly within the plate away from the upper right margin. Lists are negligible. Length 44–58 µm, transdiameter 39–51 µm. This species is one of several large, subspherical species. Its asymmetry, wide first apical plate, small ventral pore located within the plate and lack of significant sulcal lists, clearly distinguishes it from other similar species such as *A. kutnerae.*

Distribution: Korea (Jinhae Bay), Gulf of Thailand, Manila Bay (Philippines) and Viet Nam.

15.5.3.8 *Alexandrium minutum* Halim 1960 (Fig. 15.21)

Synonyms: *A. ibericum* Balech, *A. lusitanicum* Balech

A small, widely distributed species occurring singly, varying in shape from rounded (including the antapical profile) to elongate with some antapical flattening. The first apical plate usually does not appear to contact the APC but Balech (1995) reports the presence of a fine anterior projection from the first apical homologue reaching the APC. In some cells the main part of the first apical does have a narrow contact with the APC. The anterior right margin is somewhat concave. A ventral pore is present. The surface is delicately porulate and may have a very faint, irregular reticulation although some have been reported to have a strong reticulation confined to the hypotheca. The anterior sulcal plate is fairly conventional for the genus, slightly wider than long. The APC is narrowly triangular with no attachment pore. Length 15–29 µm, transdiameter 13–21 µm.

Distribution: Egypt, Turkey, Italy, Spain (both in the Mediterranean and on the Atlantic coast), Portugal, France, the east coast of the USA, Taiwan, Thailand, Australia and New Zealand.

Comments: A few of the above records are for forms lacking a ventral pore and these may be confused with the closely similar species *A. angustitabulatum*.

15.5.3.9 *Alexandrium monilatum* (Howell) Balech (Fig. 15.16)

Synonyms: *Gonyaulax monilatum* Howell, *Gessnerium mochimaensis* (sic) Halim, *Gessnerium monilata* (Howell) Loeblich

A large, distinctive, chain-forming species. The cells in the chain are strongly flattened antero-posteriorly. Some angularity of the ‘shoulders’ may be present. The plates have delicate porulation. The first apical homologue does not come close to the APC (metasert) and is five-sided, wider than long, lacking a ventral pore. The APC is large and triangular, slightly curving, usually with a large anterior attachment pore near the dorsal end of the apical pore. The posterior sulcal plate is large with

relatively straight sides and a large notch where it contacts the other sulcals. A large posterior attachment pore is centrally located. Length 28–52 µm, transdiameter 33–60 µm.

Distribution: A warm-water species known from several tropical Atlantic locations (east coast of Florida, Gulf of Mexico, Venezuela) and the tropical Pacific (Ecuador).

Comments: This species is a powerful fish killer. Halim, in a paper in which his drawings were optically reversed, reported a much larger size (length 57–70 µm). The species is somewhat similar to *A. pseudogonyaulax* but the latter has a ventral pore, is more rounded and has a narrower APC and a different posterior sulcal plate.

15.5.3.10 *Alexandrium ostenfeldii* (Paulsen) Balech and Tangen (Fig. 15.21)

Synonyms: *Goniodoma ostenfeldi* Paulsen, *Gonyaulax ostenfeldi* (Paulsen) Paulsen, *Protogonyaulax ostenfeldi* (Paulsen) Fraga and Sanchez, *Heteraulacus ostenfeldi* (Paulsen) Loeblich, *Gessnerium ostenfeldi* (Paulsen) Loeblich and Loeblich, *Triadinium ostenfeldi* (Paulsen) Dodge, *Gonyaulax globosa* (Braarud) Balech, *Protogonyaulax globosa* (Braarud) Taylor, *Gonyaulax trygvei* Parke

A distinctive, large, non-chained, globose species in which both the epithecal and hypothecal profiles are smoothly rounded. The first apical plate is distinctive in shape, the right upper margin abruptly concave with a large ventral pore excavated from the margin at the point of inflection. It usually contacts the APC. The cingulum is only slightly excavated. The anterior sulcal plate is wide. Length 40–56 µm, transdiameter 40–50 µm. It is mildly toxic. It is very similar to *A. peruvianum*, which is smaller and has a more smoothly curved right upper margin to its first apical plate.

Distribution: Known from Scandinavian, Icelandic, Spanish (Galicia), Washington State, British Columbian (unpublished), Russian (Kamchatka), Egyptian, New Zealand and Australian waters.

15.5.3.11 *Alexandrium tamarense* (Lebour) Balech (Fig. 15.21)

Synonyms: *Gonyaulax tamarensis* Lebour, *Gonyaulax excavatum* Braarud, *Pyrodinium phoneus*, Woloszynska and Conrad, *Protogonyaulax tamarensis* (Lebour) Taylor, *Gessnerium tamarensis* (Lebour) Loeblich and Loeblich, *Alexandrium excavatum* (Lebour) Balech and Tangen, *Alexandrium fundyense* Balech

A medium-sized species, occurring singly or in pairs. It is somewhat pentagonal in shape, usually with a slightly indented posterior profile. The left posterior lobe is often slightly larger than the right. 'Shoulders' may be apparent on the upper profile. The margins of the first apical plate are relatively straight although the upper right may be curved. The plate contacts the APC which is variable in shape, from broadly triangular to almost narrowly rectangular. An anterior attachment pore and/or posterior attachment pore may be present in pairs. A small ventral pore is usually present (absent in the *fundyense* form). The anterior sulcal plate is narrow and relatively smoothly curving. Length 22–51 µm, transdiameter 17–44 µm.

Distribution: A widespread species in European waters, including the United Kingdom. Also on both coasts of North America, Japan, Korea, Australia, New Zealand and Argentina. A tropical Atlantic record has now been referred to *A. tropicale*.

Comments: This was the first species of the genus to be described. Most strains are toxic but some produce only trace amounts. It is very similar to *A. tropicale*, which differs in having a narrow, anteriorly pointed first apical plate and is somewhat more rounded.

15.5.3.12 *Alexandrium tamiyavanichi* Balech (Fig. 15.21)

A chain-forming species, the cells being isodiametric or slightly wider than long. The plates are strongly porulated. The epitheca is wider than long, conical. The first apical plate usually has substantial contact with the APC and has a small ventral pore. The sides of the first apical are relatively straight. The APC is broad with an anterior attachment pore. The anterior sulcal has a distinctive anterior flange demarcated by a ridge. This projects into a notch in the epitheca. The posterior sulcal is rather variable in shape, with a posterior attachment pore near the centre. A strain of this species from the Gulf of Thailand has been shown to be toxic. The species is very similar to *A. cohorticula* which also forms chains, is a similar size and has the anterior flange on the anterior sulcal plate. Balech (1995) considers the key differences to be the proportions of the epitheca, details of the APC with the apical pore more ventral in the plate in *A. cohorticula*, a narrower and more irregular first apical plate in the latter and other small differences.

Distribution: Gulf of Thailand, Manila Bay.

15.5.4 *Pyrodinium* Plate

Cells with a very similar tabulation to *Alexandrium* but differing in having a much heavier theca with strong surface markings and flanged sutures. Left-handed girdle displacement. The sulcal lists create a tunnel that includes the plate conventionally considered to be the first postcingular plate. Cysts spiny.

15.5.4.1 *Pyrodinium bahamense* Plate *var. compressum* (Böhm) Steidinger, Tester et Taylor (Fig. 15.22)

Synonyms: *Pyrodinium bahamense* f. *compressa* Bohm, *Gonyaulax schilleri* Matzenauer, *Hemicystodinium zoharyi* Rossignol (cyst), *Polysphaeridium zoharyi* (Rossignol) Bujak *et al.*

Cells single or, more commonly, in chains. Single cells rounded, those in chains showing distinct apico-antapical flattening. Thecal surface covered with dense fine spinulae (only clearly visible with scanning electron microscopy) and large, prominent pores. Strong low flanges mark the edges of most sutures, some more developed than others. The first apical plate homologue does not reach the APC (metasert). A distinct ventral pore is present near the upper right junction with the fourth apical plate. The apex is a low horn. The APC is large, triangular, with a very distinct separation of the inner and outer pore plates (spinules only on the inner). An anterior attachment pore lies close to the right margin of the inner plate. The girdle has well-developed lists. The sulcal lists are large and contact each other anteriorly. Single individuals have prominent anterior and posterior spines. In intact chains, only the anterior individual has an apical spine and only the most posterior cell has an antapical spine. The posterior sulcal plate is narrow, median, with a slit-like posterior attachment pore. Length of cell body 33–47 µm, transdiameter 37–52 µm. Cyst spiny (see Chapter 20).

Distribution: Principally in south-east Asian waters from the Philippines to New Guinea, including Palau, Solomon Islands, Halmahera, Sabah, Brunei and also northern Indian Ocean, Red Sea. Possibly the Pacific coast of Central America. The var. *bahamense* occurs in the tropical West Atlantic.

Comments: The var. *bahamense* does not form chains and so all cells in a population are roughly isodiametric with large apical and antapical spines. The pores are often not as prominent. Toxic strains have not been found so far. The sketch of the sutural relations of the first apical homologue and the APC by Steidinger (1990) erroneously shows an exsert condition (the genus is metasert, as noted above).

15.5.5 *Protoceratium reticulatum* (Claparède and Lachmann) Bütschli (Fig. 15.19)

Synonyms: *Peridinium reticulatum* Claparède and Lachmann, *Gonyaulax grindleyi* Reinecke

Oval, strongly pigmented cells with a gonyaulax-like girdle displacement. The girdle is anterior to the midpoint of the cell (hypotheca is larger than epitheca). The surface is densely reticulated so that plate sutures are hard to see in an intact cell. The first apical plate is angular with parallel long sides and a prominent ventral pore on its left side. An APC is present. Length 28–53 µm, transdiameter 25–45 µm. The cyst is spherical with capitate spines (see Chapter 20).

Distribution: Widespread temperate and subtropical coastal species, very common in the summer although blooms (see below) are rare. Its cyst is the commonest in temperate coastal sediments in many regions, including British Columbia, and it has been described as abundant in the sediments of Oslofjord.

Comments: This common species was described in the late nineteenth century. Its tabulation is obscured by the dense reticulation on the surface of its plates. Lebour showed some tabulation but almost certainly wrong in detail. Woloszynska showed the large ventral pore on the first apical plate and reasonably accurate tabulation. Reinecke provided detailed tabulation from a bloom in the Cape, South Africa, and on that basis assigned it to the broadly defined genus *Gonyaulax*. The latter is now defined more narrowly, partly as a result of the recognition of differing cyst morphologies and so this taxon reverts to its earlier name. Steidinger and Tangen (1996) have noted that the genus *Protoceratium* needs clearer definition (the type, *P. aceros*, has not been clearly described since it was first named). There are a few tabulational questions that need to be resolved and some authors prefer *G. grindleyi*. Producer of yessotoxin.

15.5.6 *Gonyaulax* Diesing

From the older literature it might appear that several species of this genus are toxic or harmful in other ways. This is partly due to the original inclusion of several *Alexandrium* species in this genus. An early report of the production of PSP toxin by *Gonyaulax polyedra* (now commonly placed in the separate genus *Lingulodinium* as *L. polyedrum)* has not been substantiated. Several species of *Gonyaulax*, such *G. polygramma* and *G. reticulatum*, have been implicated in marine fauna mortalities in Hong Kong, False Bay (South Africa) and elsewhere, but these appear to be non-specific deaths due to oxygen depletion and any strongly blooming species of this genus could probably produce kills under the right circumstances.

15.5.7 *Gambierdiscus* Adachi et Fukuyo

This highly flattened genus has a tabulation that is fundamentally similar to *Pyrodinium* and *Alexandrium*, even though it looks very different. The first apical homologue is small and exsert (referred to as a precingular by some authors). Initially only one species, *Gambierdiscus toxicus*, which is flattened and benthic, was described by light microscopy (Adachi and Fukuyo, 1979). Scanning electron microscopy was subsequently used to describe *G. belizeanus* Faust with areolate thecal ornamentation and *G. yasumotoi* Holmes, which is globular in shape like *Coolia*. Combined morphological and molecular classification has also allowed the discrimination of *G. australes* Faust and Chinain, *G. pacificus* Chinain and Faust and *G. polynesiensis* Chinain and Faust (Chinain *et al.*, 1999; Faust, 1995; Holmes, 1998). *Gambierdiscus* is believed to be the primary source of ciguatoxin and maitotoxin (see Chapter 10).

15.5.7.1 *Gambierdiscus toxicus* Adachi et Fukuyo (Fig. 15.23)

This large species is strongly flattened apico-antapically. In apical view it is round to broadly ovoid, sometimes somewhat squarish in outline with a distinct sulcal indentation. In ventral view it is lenticular and the girdle appears to be displaced in a curving right-handed way. The sulcus is deeply impressed and pocket-like. Lists are not present. The plates are densely poroid. The APC is triangular with a hook-like apical slit. The APC is inclined towards the left of the cell. Although usually sessile on seaweeds, the cells can swim when disturbed. Length 24–60 µm, transdiameter 42–140 µm.

Distribution: Circumtropical species, found on seaweeds in sheltered coral reef areas away from strong land runoff. Small, dry islands and offshore banks and reefs are preferred.

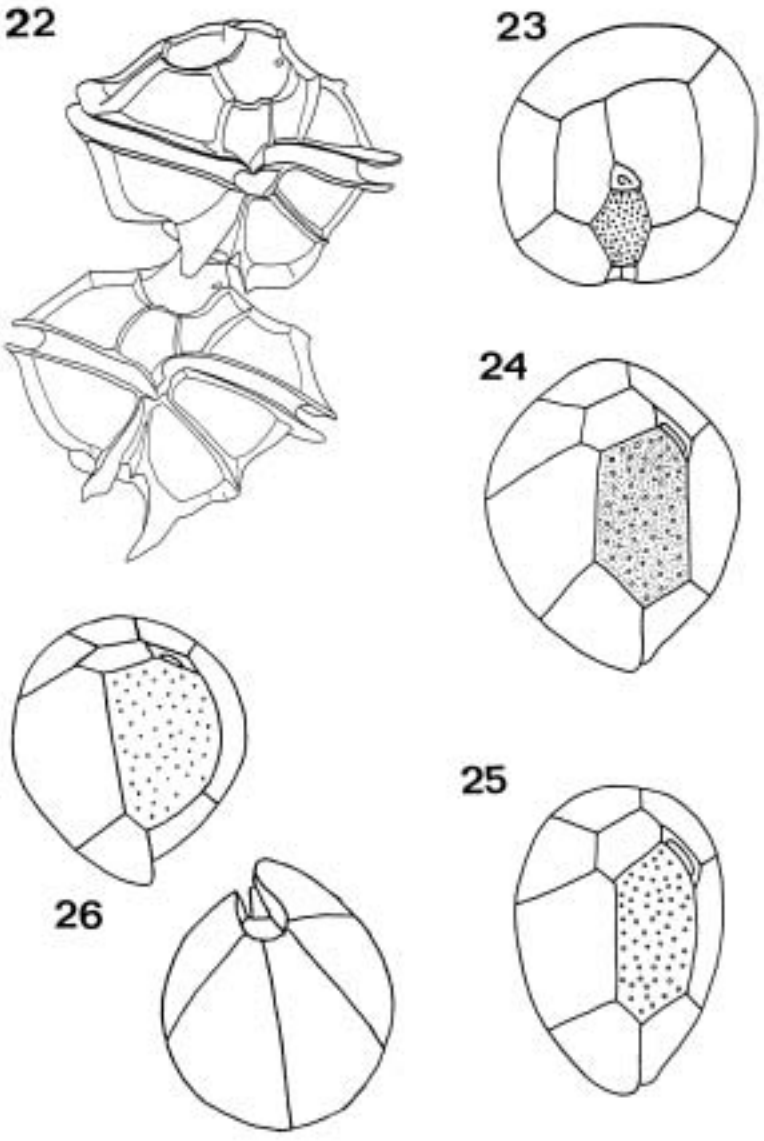
15.5.8 *Ostreopsis* Schmidt

Another large, strongly flattened benthic genus with a tabulation fundamentally similar to *Gambierdiscus* but with a high degree of distortion due to dorso-ventral elongation. The APC is narrow and displaced dorso-laterally to the left. The cells are elliptical and pointed towards the sulcus and there is no obvious sulcal notch when seen in broad view (unlike *Gambierdiscus* with which it might be confused under low magnification). The species are all epibenthic, often attached to the substrate by a fine thread, the flagella beating while tethered in this manner. Generally less substrate specific than *Gambierdiscus*. Currently at least nine morphospecies are distinguished, *O. siamensis* Schmidt, *O. lenticularis* Fukuyo, *O. ovata* Fukuyo, *O. heptagona* Norris, *O. mascarenensis* Quod, *O. labens* Faust and Morton, *O. marinus* Faust, *O. belizeanus* Faust and *O. caribbeanus* (Faust, 1999). These species differ in fine details of thecal plate size and shape, and thecal pore ornamentation.

15.5.8.1 *Ostreopsis lenticularis* Fukuyo (Fig. 15.24)

Cells roundly lenticular, slightly pointed towards the sulcus. Plates covered with large pores as well as numerous fine pores. The girdle does not undulate and there is no girdle displacement. Dorso-ventral distance 60–100 µm, transdiameter 45–80 µm. Distinguished from *O. siamensis* chiefly by lack of undulation of the girdle and two types of pore.

Distribution: Probably circumtropical, benthic species. Currently known from French Polynesia (Gambier Islands), New Caledonia and several tropical Atlantic localities including Cayman Islands, Puerto Rico and the Virgin Islands.



Figures 15.22–26
22, *Pyrodinium bahamense* var. *compressum*;
23, *Gambierdiscus toxicus*;
24, *Ostreopsis lenticularis*;
25, *Ostreopsis siamensis*;
26, *Coolia monotis*.
Sources: 22, Yoshida;
23–26, Fukuyo.

Comments: The report of ostreopsis-toxin by this species appears to have been based on cultures of *O. siamensis.*

15.5.8.2 *Ostreopsis siamensis* Schmidt (Fig. 15.25)

Cells ovoidal in apical view, some rounder than others, pointed towards the sulcus. In side view the cell undulates somewhat and the girdle also undulates. The plates are covered with large pores. Similar in size to *O. lenticularis.* Dorso-ventral distance 60–100 µm, transdiameter 45–90 µm. A producer of both fat- and water-soluble toxins, including palytoxins and its analogues.

Distribution: Species first described from the Gulf of Thailand. Subsequently found at numerous Pacific Island locations, the tropical Caribbean and the Mediterranean. Similar forms are also known from temperate habitats in New Zealand and Australia (Rhodes *et al.*, 2000).

15.5.8.3 *Coolia monotis* Meunier (Fig. 15.26)

Globular cells, 21–40 µm diameter, slightly compressed anterio-posteriorly. Cingulum descending but appearing straight in lateral view. The apex is displaced dorsally and to the left. In apical view, the epitheca is smaller than the hypotheca. Fan-shaped arrangement of postcingular plates, The hypothecal plate pattern is different than in *Ostreopsis*. *C. monotis* produces yessotoxin analogues.

Distribution: Worldwide, planktonic, benthic and epiphytic species, from temperate to tropical waters.

Comments: Coolia tropicale Faust differs in having a large wedge-shaped first apical plate in the centre of the hypotheca.

15.5.9 *Ceratium* Schrank

A gonyaulacoid genus characterized by the presence of two or three hollow horns (four in some freshwater species) and a broad, window-like depression on the ventral side. Although not shown to produce toxins, the common species below has been linked to oyster larval mortality. *Ceratium tripos* has been linked to several anoxic or hypoxic events.

15.5.9.1 *Ceratium fusus* (Ehrenberg) Dujardin (Fig. 15.20)

Synonym: *Peridinium fusus* Ehrenberg, several others from the nineteenth century

Like other members of the Section Fusiformia, the cells are very elongate with two long, slightly and smoothly curved horns, the apical and left antapical horns. The right antapical horn, well developed in many *Ceratium* species, is greatly reduced or completely absent. Length 150–300 µm, transdiameter 15–30 µm. Can cause harm to invertebrate larvae by an unknown mechanism.

Distribution: Very widespread in coastal waters, where the species can tolerate a great range of salinities (5–70 ppt).

15.6 Dinophysoids

Members of this group are the only thecate dinoflagellates fundamentally divisible into two lateral halves and possess a girdle (very anterior in position) and a short sulcus.

They are laterally compressed. and their shapes in lateral view are most important for genus and species identification. Two families, Dinophysiaceae and Amphisoleniaceae, with about 15 genera, are known. Most of their members, with the exception of those belonging to the genus *Dinophysis*, are rather rare species in tropical and temperate dinoflagellate communities, and never cause water discolorations. Toxins causing diarrhetic shellfish poisoning were detected in seven *Dinophysis* species, but toxicity of other dinophysioids has not yet been studied.

15.6.1 *Dinophysis* Ehrenberg

Synonyms: *Phalacroma* Stein, *Dinoceras* Schiller, *Prodinophysis* Balech

Dinophysis is one of the largest genera of dinoflagellates with more than 200 species described. The genus name *Phalacroma* had been used for members possessing a large convex epitheca. Abe (1967) and Balech (1967) independently observed micromorphology of the sulcal area and concluded to abandon the junior synonym *Phalacroma* due to lack of significant differences of taxonomic characteristics. However, it has been pointed out that most *Phalacroma* are heterotrophic, oceanic species and most *Dinophysis* are photosynthetic and/or mixotrophic, coastal organisms (Hallegraeff and Lucas, 1988).

For species identification of *Dinophysis*, important features are size and shape of the cells, especially outline of the main body and sulcal lists, and presence or absence of chloroplasts. Thecal plate configuration and morphological characteristics of plates themselves may have importance for taxonomy, but it is premature to separate species on the basis of minor variations in some thecal plates.

Dinophysis reproduce asexually by longitudinal binary fission. After the fission, two daughter cells having an incomplete sulcal list often attach at their dorsal margin. The sexual reproduction process is not yet well known. A small-sized species simultaneously occurring with a larger-size species may be a gamete of the latter. This phenomenon is found in *D. lapidostrigiliformis* with *D. fortii* (Fukuyo *et al.*, 1981), *D. dens* with *D. acuta*, *D. diegensis* with *D. caudata* or *D. tripos*, and *D. skagii* with *D. acuminata* or *D. sacculus* (Moita and Sampayo, 1993; Reguera and Gonzales-Gil, 2001). Close contact of the small species with the large one at the ventral side, often followed by engulfment of the small into the large cell, is suspected to be a process of sexual fusion.

Productivity of toxins responsible for diarrhetic shellfish poisoning, okadaic acid or its derivative dinophysistoxins (see Chapter 8) or both, has been confirmed in seven species: *D. acuminata, D. acuta, D. fortii, D. mitra, D. norvegica, D. rotundata* and *D. tripos* (Lee *et al.*, 1989). Three other species, *D. caudata, D. hastata*, and *D. sacculus*, are also suspected. The number of toxic species will probably increase in the future, but the confirmation of their toxigenicity is not easy because of the difficulty in obtaining *Dinophysis* clonal cultures. Lee *et al.* (1989) confirmed the toxicity in 100–1,000 cells of the above-mentioned species, using specimens collected from natural plankton samples under microscope by capillary manipulation. Toxin productivity varies considerably among species, and among regional and seasonal morphotypes in one species. For example, *D. fortii* in northern Japan during March and June contains high concentrations of toxins and is associated with significant accumulation of toxins in shellfish. But the same species in southern Japan during May and July does show slight toxicity and shellfish is free from toxins. In a strict taxonomic sense, a higher percentage of *D. fortii* cells in southern Japan have

more convexed curvature in dorsal outline, but this morphological difference is not sufficient to distinguish northern and southern morphotypes (high and low toxin producers, respectively). Therefore clonal differences in toxin productivity, associated with minor morphological variations in a single species, are often found in *Dinophysis*.

15.6.6.1 *Dinophysis acuminata* Claparède et Lachmann (Fig. 15.27)

Synonyms: *D. lachmannii* Paulsen, *D. borealis* Paulsen, *D. boehmii* Paulsen

Cells oval or often narrow and elongated oval in lateral view. Dorso-ventral depth longest near the middle, about half of the cell length. Epitheca low, flat or weakly convex, invisible in lateral view. Dorso-ventral depth of epitheca is 1/3 to 1/2 of hypotheca. Antapex is rounded and smooth, or with two to four knob-shaped small protuberances. Left sulcal list rather narrow, often coarsely areolate, supported by three ribs, extending to 1/2 to 2/3 of cell length. Thecal plates thick, areolated. Length 40–50 µm, dorso-ventral depth 30–40 µm.

Distribution: This species is distributed widely in temperate waters and seems to appear abundantly in coastal waters, especially eutrophic areas. Blooms are often associated with toxification of shellfish (see Larsen and Moestrup, 1992).

Comments: As size and shape varies considerably, differentiation of *D. acuminata* from morphologically similar species having 'acuminated' features is rather difficult. Paulsen (1949) established three new species; *D. borealis*, *D. lachmannii* and *D. skagii*. Solum (19623) observed that morphological variation among the species was mostly caused by environmental salinity differences and contained no discontinuities. Furthermore Abe (1967) and Balech (1976) re-accommodated *D. borealis* and *D. lachmannii* in *D. acuminata*. *D. skagii* is also considered as an aberrant form (Dodge, 1982). Specimens collected from northern France (shown in Lassus and Bardouil, 1991) and Norwegian waters (shown in Balech, 1976) have a straighter and flatter ventral outline than those in Japanese waters (shown in Abe, 1967, and Fukuyo *et al.*, 1990). The antapex moves ventrally and is more or less pointed in European cells, whereas Japanese ones have the antapex at the middle and a rounder posterior contour. Similar to *D. fortii*, toxicity of *D. acuminata* varies considerably between seasons and areas where it blooms.

15.6.6.2 *Dinophysis acuta* Ehrenberg (Fig. 15.28)

Cells large, strongly flattened laterally. In lateral view, cells ovoid with pointed or somewhat rounded posterior end. The posterior third of the hypotheca shows a triangular shape with the posterior end moving slightly ventrally. Epitheca low, flat or weakly covex, invisible in lateral view. Dorso-ventral depth largest below the middle, almost at 2/3 of total length, about 1/2 to 2/3 of cell length. Dorso-ventral depth of epitheca is 1/2 to 2/3 of hypotheca. Left sulcal list wider posteriorly, supported by three ribs, extending about 2/3 of cell length. Thecal plates thick, coarsely areolated. Length 54–94 µm, dorso-ventral depth 43–60 µm.

Distribution: This species is distributed widely in cold and temperate waters. Blooms are associated with toxification of shellfish (see Larsen and Moestrup, 1992).

Comments: This species looks similar to *D. norvegica*, but can be differentiated by the larger size and posteriorly located deepest position of the former species. Moita and Sampayo (1993) reported a gamete, which was identified as *D. dens*, and a resting cyst in natural plankton samples during blooming season of *D. acuta*. As

the morphological character of the cyst was very different from those of other dinoflagellates, confirmation by culture experiments is necessary.

15.6.6.3 *Dinophysis caudata* Saville-Kent (Fig. 15.29)

Synonym: *D. homunculus* Stein

Cells large, irregularly subovate with fairly distinctive long ventral projection. Ventral side of hypotheca undulate or straight. Dorsal side straight or slightly concave in the anterior half, and straight or convex, running parallel with the ventral side in the posterior half.

Anterior cingular list wide, supported by many ribs, forming a wide and deep funnel-like structure with very low epitheca on the bottom. Left sulcal list almost half of total length, supported by three ribs. Thecal plates thick, areolated. Length 70–110 µm.

Distribution: This species is distributed widely in tropical and temperate waters and seems to appear abundantly in coastal waters. Red tides associated with mass mortality of fish were reported in the Gulf of Thailand and Seto Inland Sea in Japan (Okaichi, 1967).

Comments: Cell shape, especially length of ventral projection and extent of dorsal expansion, is rather variable. Cells with a long projection usually have wide sulcal lists and well-developed cingular lists, and differentiation from *D. tripos* becomes difficult. On the contrary, cells with short projection look similar to *D. diegensis*, which is suspected to be a gamete of the species (Moita and Sampayo, 1993). Toxicity of the species was found using specimens collected in Philippine waters (Kodama, pers. comm.).

15.6.6.4 *Dinophysis fortii* Pavillard (Fig. 15.30)

Cells large, subovate with fairly straight ventral side in the anterior half, and broadly rounded posterior. Dorsal side smoothly convex with slight concavity near the cingulum. Dorso-ventral depth of epitheca is about 1/2 of hypotheca. Dorso-ventral depth largest below the middle at the base of the third rib. Anterior cingular list forms a wide and low cup-like structure with very low epitheca at the bottom. Sulcal list very long, supported by three ribs, extending to 4/5 of cell length. Reticulation on the list is obvious. Thecal plates thick, areolated. Length 60–80 µm.

Distribution: This species is distributed widely in temperate waters. In northern Japan brought by a warm current in spring and early summer, propagating in coastal areas where shellfish aquaculture is intensive.

Comments: Many scientists have tried to establish cultures of *D. fortii* using several kinds of enriched seawater medium, but could keep at most 30 cells/ml for several months. The inoculated cells did not die, but reproduced very slowly. As mean doubling time is reported as 1.4 days in natural conditions (Weiler and Chisholm, 1976), environmental factors for rapid growth must be overlooked in experimental incubations. The species is most noxious by its productivity of DTX1, PTX2 and OA, but clones in warmer waters often show very low toxicity.

15.6.6.5 *Dinophysis miles* Cleve (Fig. 15.31)

Cells very large, anterio-posteriorly elongated with two fairly distinctive, long, antapical and dorsal projections. Ventral side of hypotheca undulate. Dorsal side concave and smoothly continues to the dorsal projection, which runs obliquely backwards. The distal end bends at a right angle, carrying a wing-like unabsorbed

remnant of the megacytic zone. Six to eight daughter cells often attach at the remnant after asexual cell division. Posterior projections shorter or longer than, or as long as, the dorsal process. Angle between the dorsal and posterior projections 50–90°C, beginning at the base of the third rib. Anterior cingular list wide, supported by many ribs, forming a narrow funnel-like structure with very low epitheca on the bottom. Thecal plates thick, round or angular areolated. Length 125–150 µm. This species is easily identified by its large cell shape and especially the two projections. Kodama (pers. comm.) found toxicity of the species using specimens collected in Philippine waters.

Distribution: This species is distributed widely in tropical waters.

15.6.6.6 *Dinophysis mitra* (Schutt) Abe (Fig. 15.32)

Synonym: *Phalacroma mitra* Schutt

Cells broad and wedge-shaped. Dorsal side smoothly convex. Ventral side flat or somewhat undulate in anterior half, and slightly concave in posterior half. Epitheca flat or slightly convex, broad ellipsoidal in apical view. As the megacytic zone becomes wide due to cell growth, the epitheca becomes round in apical view and the posterio-ventral concavity becomes much less distinct. Dorso-ventral depth of epitheca and hypotheca almost the same and longest at the base of the second rib. Cingulum located most anteriorly. Both anterior and posterior cingular lists narrow, supported by many ribs. Sulcal list rather short, about half of the total length, supported by three short ribs. Thecal plates thick, coarsely areolated. Length 56–68 µm, width 48–58 µm. This species resembles *D. rapa*, but can be distinguished by the more strongly protuberant sulcal ridge at the base of the third rib and distinctive concavity at the posterio-ventral hypotheca of the latter species (Abe, 1967). The production of DTX1 toxin was confirmed by Lee *et al.* (1989).

Distribution: This species is distributed widely in temperate waters.

15.6.6.7 *Dinophysis norvegica* Claparède and Lachmann (Fig. 15.33)

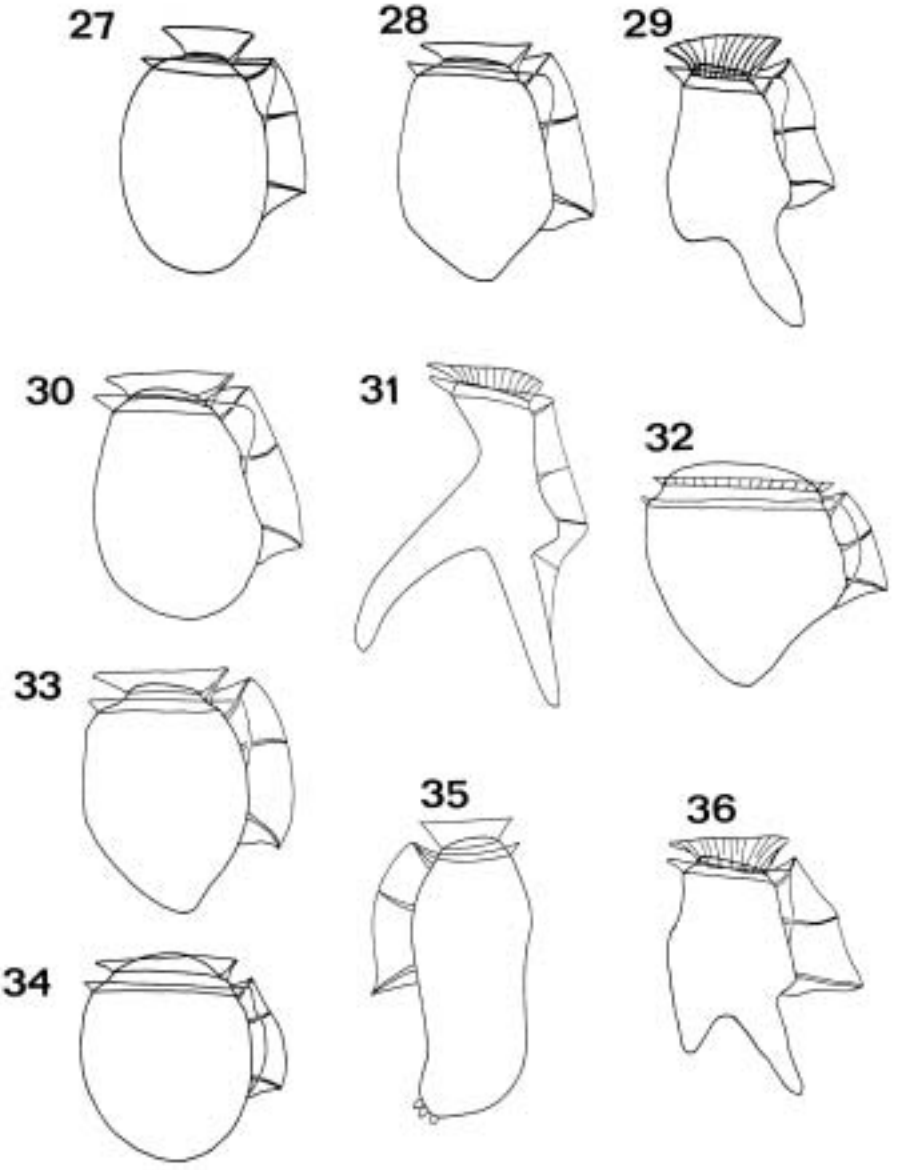
Cells strongly flattened laterally, ovoid with pointed or somewhat rounded posterior end in lateral view. Dorsal side smoothly convex, often with a small protuberance. Posterior half of hypotheca tapers to a triangular shape. Posterio-ventral side straight or slightly concave. Epitheca low, flat or weakly covex, invisible in lateral view. Dorso-ventral depth longest at the middle, between the bases of the second and third rib. Dorso-ventral depth of epitheca is 1/2 to 2/3 of hypotheca. Left sulcal list often undulated, supported by three ribs, extending about 2/3 of cell length. Thecal plates thick, coarsely areolated. Length 48–80 µm, dorso-ventral depth 39–70 µm. This species looks similar to *D. acuta*, but can be differentiated by its size and deepest position (see comment on *D. acuta*). DTX1 and OA production was reported by Lee *et al.* (1989).

Distribution: This species is distributed widely in cold and temperate waters.

15.6.6.8 *Dinophysis rotundata* Claparède and Lachmann (Fig. 15.34)

Synonyms: *Phalacroma rotundatum* Kofoid and Michnener, *Dinophysis whittingae* Balech

Cells asymmetrically round-oval in lateral view, and ellipsoidal to round in apical view. Epitheca low and fairly evenly rounded, convex, and visible laterally. Both anterior and posterior cingular lists narrow, lacking any ridges. Sulcal list also narrow, extending about 1/2 to 3/5 of total length. The list has three ribs, among which



Figures 15.27–36
Dinophysoid dinoflagellates.
27, *Dinophysis acuminata*;
28, *D. acuta*; 29, *D. caudata*;
30, *D. fortii*; 31, *D. miles*;
32, *D. mitra*; 33, *D. norvegica*; 34, *D. rotundata*;
35, *D. sacculus*; 36, *D. tripos.*
Sources: Inoue, except 35, Yoshida.

the second is located nearer the first. Dorso-ventral depth is largest between base of second and third ribs. Thecal plates thick, finely areolated. Protoplasm does not contain chloroplasts, but often has many large food vacuoles. Length 42–50 µm, dorso-ventral depth 36–43 µm.

This species looks similar to *D. rudgei*, but is distinguishable by the low height of the epitheca of the former species. The epitheca of *D. rudgei* is quite easily visible in lateral view. *Dinophysis rotundata* is a phagocytic species, feeding on both loricated and non-loricated ciliates by piercing the prey cell with a feeding tube (Hansen, 1991; Inoue *et al.*, 1993). This is the first heterotrophic dinoflagellate in which toxin productivity has been confirmed (Lee *et al.*, 1989).

Distribution: This species is distributed widely in cold and temperate waters.

15.6.6.9 *Dinophysis sacculus* Stein (Fig. 15.35)

Cells irregular long oval. Dorsal side straight or undulating: convex below the cingulum, slightly concave in the middle, and convex again posteriorly. Ventral side also undulate: convex at the middle, concave below the middle. Anterior cingular list low without distinctive ribs. Dorso-ventral depth of epitheca is 1/3 to 1/2 of hypotheca. Deepest part of dorso-ventral width varies because of undulation of both dorsal and ventral side, but mostly deepest at between the second and third rib. Sulcal list rather short, about 1/2 of cell length, reaching the middle of hypotheca. Thecal plate coarsely areolated. Length 40–60 µm.

Although toxin production has not been confirmed, this species is considered to be responsible for DSP toxin occurrence in bivalves in Portuguese coastal waters (Alvito *et al.*, 1990; Sampayo *et al.*, 1990).

Distribution: This species is distributed widely in cold and temperate waters.

15.6.6.10 *Dinophysis tripos* Gourret (Fig. 15.36)

Cells large, anterio-posteriorly elongated with distinctive long antapical and short dorsal projections. Ventral side of hypotheca straight or slightly undulate. Dorsal side concave below the cingulum and then convex, smoothly continuing to the dorsal projection which runs backwards. The posterior end of both projections often bears several minute protuberances.

Anterior cingular list wide, supported by many ribs, forming a narrow funnel-like structure with very low epitheca on the bottom. Sulcal list extends widely posteriorly with reticulation. Thecal plates thick, coarsely areolated. Length 90–125 µm. Easily identified by its two projections. Lee *et al.* (1989) found DTX1 toxin in this species.

Distribution: D. tripos is distributed widely in tropical and temperate waters, and occasionally appears in colder regions.

15.7 Prorocentroids

The species of this group are easily distinguishable from other dinoflagellates by two anteriorly inserted flagella and two large, laterally flattened valves. One of the valves, the right one, has a small indentation, which is occupied by seven or eight tiny periflagellar platelets forming a flagellar pore and an accessary pore. Two genera, *Mesoporos* and *Prorocentrum*, are known. The former has a large central pore at the centre of the valves.

15.7.1 *Prorocentrum* Ehrenberg

Synonym: *Exuviaella* Cienkowski

Classification of members of this genus is based on morphological characteristics such as presence of anterior spine, cell shape, ornamentation of valve surface (smooth, or covered with depressions or spinules), and distribution pattern of trichocyst pores. Two distinctive life forms, planktonic and epibenthic, are known. Planktonic species have been studied for a long time, and more than 60 taxa are reported under the genus name of either *Prorocentrum* or *Exuviaella*. Dodge and Bibby (1973) combined the two genera after careful morphological observation using electron microscopy, which was followed by revision of taxonomy with reduction of species numbers from 64 to 21 by Dodge (1975). Although many planktonic *Prorocentrum* species are known to form red-tide discolorations, e.g. *P. micans*, *P. cordatum (= minimum)* and *P. triestinum*, most are harmless.

In contrast to the planktonic forms, many epibenthic species (Faust 1995) are suspected to have implications in poisonings endemic in tropical areas. For species identification, architecture of periflagellar area and intercalary bands are suggested as important criteria, in addition to those used for the planktonic species.

15.7.2 *Prorocentrum concavum* Fukuyo (Fig. 15.37)

Cells broadly ovate in valve view, widest behind the middle, lenticulate to ellipsoidal in lateral view with a flattened central area. The anterior end of left and right valves is straight and deeply concave, respectively. Both sides of the concavity rise roundly. Valve surface covered with many depressions. Trichocyst pores distributed all over the surface except the central area, but denser near the margin. Pyrenoid at the centre and nucleus posteriorly. Length 45–55 µm, width 38–45 µm.

Comments: Most epibenthic species share ovate or ovoidal valve shape without distinctive anterior spine, and it is difficult to distinguish *P. concavum* from similar species such as *P. lima*, *P. hoffmannianum,* and *P. sabulosum*. Important and reliable criteria are the surface ornamentation of the valve and the number and configuration of trichocyst pores. The valve surface of *P. lima* is smooth, whereas that of *P. concavum* and the two above-mentioned species is areolate. The distinction among the three species can be made by number of areolae: *c.* 1,000 for *P. concavum*, *c.* 670 for *P. hoffmannianum* (Faust, 1990*b*), and *c.* 390 for *P. sabulosum* (Faust, 1994). Several toxins having different effects have been isolated (see Bomber and Aikman (1988–1989), but their implications in natural foodwebs in tropical areas are not known. This is mostly because of low population densities of the species.

Distribution: Epibenthic species on macroalgae growing on dead corals in coral reefs (Fukuyo, 1981), and on floating detritus in mangrove (Faust, 1990b) in tropical and subtropical areas.

15.7.3 *Prorocentrum cordatum* (Ostenfeld) Dodge (Fig. 15.38)

Synonyms: *Exuviaella cordata* Ostenfeld, *Prorocentrum minimum* (Pavillard) Schiller, *Exuviaella marina* Pavillard, *Prorocentrum triangulatum* Martin, *E. mariae-lebouriae* Park et Ballantine, *P. cordiformis* Bursa, *P. mariae-lebouriae* (Park and Ballantine) Loeblich III

Cell heart-shaped, triangular, or ovate in valve view, lenticular in lateral view. Anterior margin straight or slightly convex with a small depression at the middle. A short

spine rises from a side of the depression. In the original description of *Exuviaella cordata* from the Caspian Sea it was claimed that this taxon lacked an apical spine, which was used to distinguish it from *Exuviaella minima* (type locality Gulf of Lion). A recent SEM study of Caspian Sea material revealed the presence of a small spine in all material studied, indicating the conspecificity of those two taxa (Velikova and Larsen, 1999). Valve surface covered with many spinules. Trichocyst pores present. Chloroplast yellowish-brown, two in number, located peripherally. Nucleus spherical to ellipsoidal, located posteriorly.

The shape of this species is so variable that several names, either at a variety or specific rank, have been given. The morphological variation, mostly due to differences in environmental parameters such as light intensity, salinity and temperature, is well observed at the curvature of lateral outline in ventral view. This species is often described as the causative organism of poisonings occurred by eating oysters and short-necked clams. The toxic substance was named as venerupin, but the chemical structure and property has not been elucidated (Hashimoto, 1979). To date, six poisoning cases have been reported in Japan and in total 542 people have fallen ill, with 185 fatal cases (34% fatality rate) (Hashimoto, 1979). As *P. cordatum* was observed abundantly in the digestive organs of toxic short-necked clams, the species was thought to be responsible (Nakazima, 1968). But observations and laboratory experiments made by Nakazima (1965*a*, 1965*b*, 1965*c*) were considered insufficient to conclude that *P. cordatum* was the causative organism (Hashimoto, 1979). Okaichi and Imatomi (1979) failed to detect venerupin in a culture of *P. cordatum*, although they could extract two substances having mouse toxicity. It is recommended to pay attention to shellfish toxicity if a bloom of *P. cordatum* affects areas for shellfish aquaculture and collection. But it is not necessary to consider *P. cordatum* as a persistent toxic organism. A recent study has shown that senescent cultures can produce chemicals with i.p. toxicity to mice (Grzebyk *et al.*, 1997).

Distribution: Planktonic species with worldwide distribution, in both marine and brackish waters.

15.7.4 *Prorocentrum emarginatum* Fukuyo (Fig. 15.39)

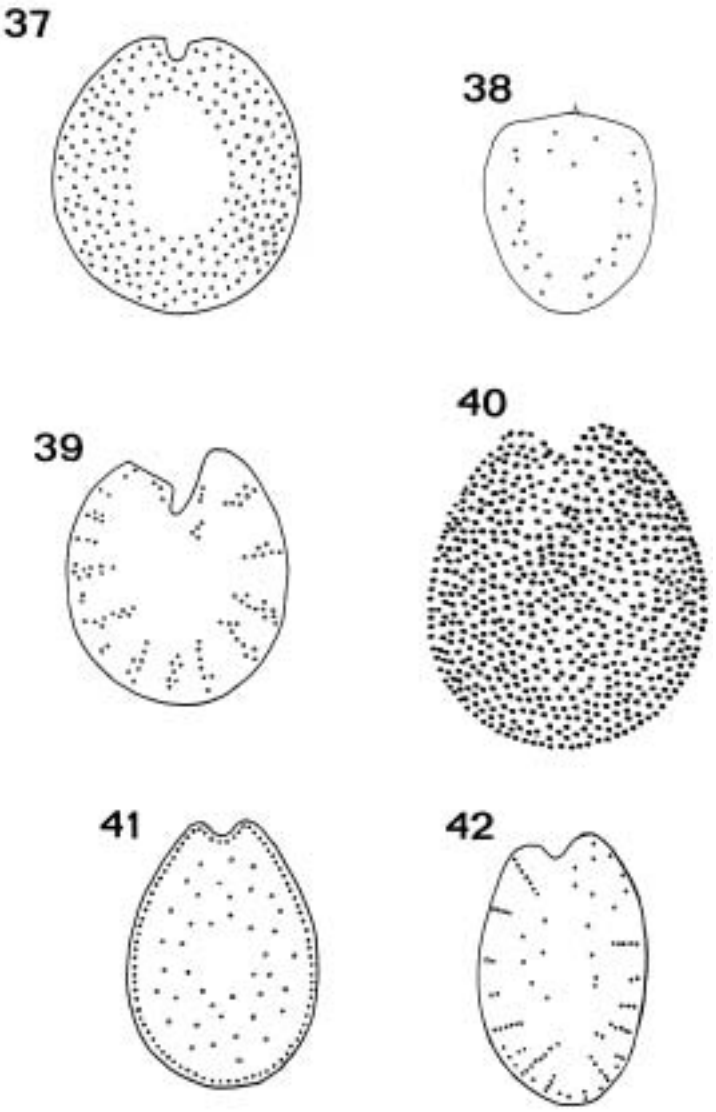
Cells ovate in valve view, lenticulate to ellipsoidal in lateral view with a flattened central area. Anterior margin widely concaved and both sides of the concavity rise into a sharp point. A wide low list stands dorsally in the concavity. The right valve has long, wide, cuneiform indentation which is curved slightly dorsally. Length 35–40 µm, width 32 µm.

The species is readily distinguishable from other benthic *Prorocentrum* except *P. sculptile* by its deep indentation at the anterior margin. Valve outline of *P. sculptile* is the same as *P. emarginatum*, but the former has round to oval depressions all over the valve surface. The former species is a little smaller (30–37 µm) than the latter (Faust, 1994). The toxicity of the species is unknown.

Distribution: Epibenthic species on macroalgae growing on dead corals in coral reefs (Fukuyo, 1981) in Ryukyu Islands, Japan.

15.7.5 *Prorocentrum hoffmannianum* Faust (Fig. 15.40)

This species, 45–44 um long, 40–45 um wide, was described from mangrove habitats at Belize, Central America. This species is larger and broader than *P. lima* and



Figures 15.37–42
Prorocentroid dinoflagellates.
37, *P. concavum*;
38, *P. cordatum*;
39, *P. emarginatum*;
40, *P. hoffmannianum*;
41, *P. lima*;
42, *P. rhathymum*.
Sources: Fukuyo, except 40, Faust.

also differs in that the valve surface is deeply areolated. Both valves are concave. The apical area is a broad triangle with a flared apical collar adjacent to the flagellar pore. The cell has a centrally located pyrenoid. Producer of okadaic acid (Faust *et al.*, 1999).

15.7.6 *Prorocentrum lima* (Ehrenberg) Dodge (Fig. 15.41)

Synonyms: *Exuviaella marina* Cienkowski, *Prorocentrum marinum* (Cienkowski) Dodge and Bibby

Cell ovate, widest behind the middle in valve view, lenticulate to ellipsoidal in lateral view with a flattened central area. The anterior end of left and right valves is straight and triangularly concave, respectively. Valve surface smooth, having trichocyst pores sparsely all over the surface except the central area. Length 30–40 µm, width 26–30 µm.

Distribution: This species is distributed very widely in tropical waters such as French Polynesia and the Caribbean Sea, to subantarctic waters such as the Swedish coast and northern Japan. Habitat is epibenthic on seaweeds and benthic embedded shallowly in sand.

Comments: Faust (1991) observed morphological characteristics of the species in detail, and suggested its wide variability of shape and size. But *P. lima* is distinguishable by its smooth valve surface from other *Prorocentrum* species, especially from *P. hoffmannianum*, which has an areolate surface. *Exuviaella marina (P. marinum)* is considered as a junior synonym of *P. lima* by Dodge (1975), but some taxonomists still treat the former as an independent *Prorocentrum* species. Sexual and asexual reproductive cycles of *P. lima* were observed by Faust (1993*a*, 1993*b*). The mature hypnozygote (resting cyst) was 70 µm in diameter, spherical, pale yellow, with a smooth, organic, triple-layered wall (Faust, 1993*a*). The same author found similar resting cysts of *P. marinum* from the same mangrove area (Faust, 1990*a*). This species produces several kinds of toxin, such as okadaic acid (see Bomber and Aikman, 1988–89), and sometimes is called a ciguatera-causing (Faust, 1991) or DSP-causing organism (Jackson *et al.*, 1993), but the implication of the toxin in food webs is unclear.

15.7.7 *Prorocentrum rhathymum* Loeblich, Sherley et Schmidt (Fig. 15.42)

Cell oval, widest anterior or at the middle in valve view, lenticulate to ellipsoidal in lateral view. Anterior end slightly concave at the middle. Low list rising from periflagellar plate looks like a short spine. Valve surface smooth, having trichocyst pores lying in rows radially from the centre perpendicular to the valve margin. Length 30–40 µm, width 22–25 µm.

Distribution: This species is distributed very widly in tropical to temperate waters. Habitat is epibenthic on seaweeds and planktonic (*rhathymum* means inactive or lazy).

Comments: This species has often been erroneously considered to be synonymous with *Prorocentrum mexicanum* Tafall, which however is planktonic, has a three- (sometimes two-) horned spine and a different distribution of trichocyst pores (present in both valves in *P. mexicanum*, but only on the left valve in *P. rhathymum*) (Cortes-Altamirano and Sierra-Beltran, 2002). Hemolytic toxin and fast-acting toxins were detected in culture of the species (Bomber and Aikman, 1988–89).

APPENDIX 15.1

Technique for the observation of dinoflagellate thecal plates

Almost all genera of armoured dinoflagellates are classified according to thecal plate configuration. For some species, thecal plate ornamentation such as smooth, mesh or porous surfaces, with or without spines, provide useful information allowing critical identification. Therefore, the observation of thecal plate characteristics is unavoidable for identification of armoured dinoflagellates, but such observation is sometimes very difficult because of the transparency of thecal plates. Modification of the regular light-microscope observation method (Fig.15.43) is thus necessary. Three such modified methods are given below.

The first method is utilization of a type of microscope that has phase contrast or differential interference contrast illumination. This enhances fine differences in thecal plate condition into a clear picture with gradations of black/white or colour. However the attachments are rather expensive, sometimes costing more than the microscope itself.

The second method is staining of thecal plates (Fig. 15.44). Von Stosch (1969) developed a useful staining solution for thin thecal plates, but it contains strong acid that causes rusting of microscope lenses. Imamura and Fukuyo (1987) modified the composition of the stain to avoid this unpleasant side effect, thus allowing wide use of the method without costly microscope repairs. The composition of the IF (Imamura and Fukuyo) staining solution and stepwise procedure is as follows:

- 2.6 g iodine
- 5.0 g potassium iodine
- 4.0 g chloral hydrate
- 10 ml distilled water.

1. Prepare full-, half- and quarter-strength solutions by diluting with distilled water. Each solution of 10 ml may last for several years and should be kept in a dark place.
2. Isolate the cell in question from a plankton sample by capillary pipette under the microscope. To avoid contamination by other species, a single cell must be sucked carefully (Fig. 15.44, step 1).
3. Place the isolated cell in a droplet of filtered seawater on a glass slide (Fig. 15.44, step 2)
4. Carefully place a cover slip over the droplet so as not to lose the cell. If the cell has no protoplasm, proceed to step 5. If the cell has protoplasm that disturbs observation, thecal plates must be separated. Add a droplet of *c.* 5% sodium hydrochlorite solution along one margin of the cover slip. At the same time, use a paper towel to draw excess water from the opposite margin, to help penetration of the solution (Fig. 15.44, step 3). All processes should be carried out carefully under the microscope.
5. Crush the cell by gently pressing the cover slip with a needle (Fig. 15.44, step 4). Thecae may open along suture lines and separate into fragments that contain several thecal plates. If the 5% solution is too strong, all the plates may detach completely and analysis of plate configuration becomes very difficult. Therefore, based on experience, the strength and amount of sodium hydrochlorite solution may need to be changed.

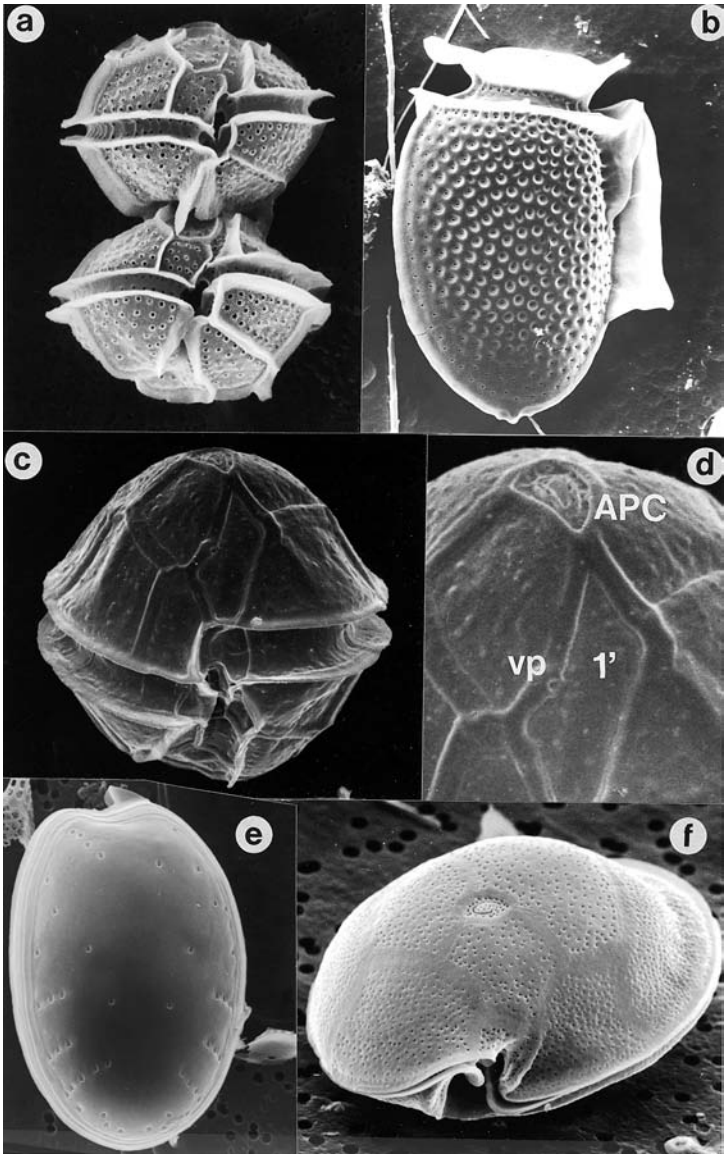


Figure 15.43
SEM of selected armoured dinoflagellates.
A, *Pyrodinium bahamense* var. *compressum* from Papua New Guinea; B, *Dinophysis acuminata* from Tasmania; C, *Alexandrium minutum* from South Australia; D, Detail of cell C, showing apical pore complex (APC) and first apical plate (1′) with ventral pore (vp); E, *Prorocentrum rhathymum* from Palau; F, *Gambierdiscus toxicus* from Great Barrier Reef, Australia.
Sources: Micrographs by Hallegraeff, except C and D, Bolch.

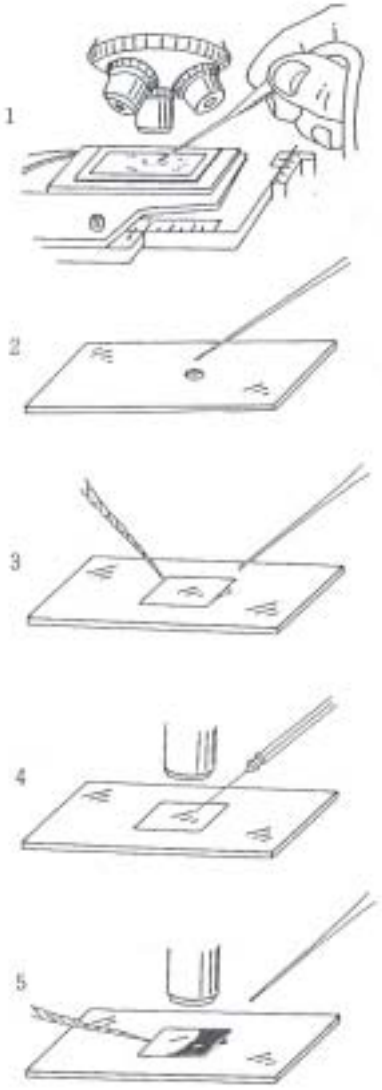


Figure 15.44
Microscopic staining protocol for dinoflagellate thecal plates.
Source: Imamura and Fukuyo (1987).

6. If the taxonomic characteristics (configuration, ornamentation) of the thecal plates are not clearly observed, add a droplet of the IF staining solution along a margin of the cover slip (Fig 15.44, step 5) and let it penetrate using a technique similar to that in step 4.
7. The stain does not last long (less than one day), as iodine colour is faded by light, thus observation and photography must be completed in a short time.

If a microscope with fluorescence capabilities is available, the observation becomes much easier. Cellufluor (Polysciences, formerly known as Calcofluor White M2R), the fluorescent stain for cellulose and other ß-linked glucans, can be used to stain the thecal plates of armoured dinoflagellates (Fritz and Triemer, 1985). Protoplasm is not stained, therefore thecal plate dissection is not required, unlike the IF staining method. Cellufluor staining allows observation of the theca lying behind protoplasm. The only disadvantage is that the fluorescence microscope is still expensive and is not routinely used for plankton work.

1. A stock solution of 10 mg ml^{-1} of Cellufluor is made with distilled water and placed in a refrigerator until use. This solution is diluted with distilled water to make a working solution with a final concentration of 0.1–100 µg ml^{-1}.
2. There are two methods for Cellufluor staining: the first is suitable for natural plankton samples preserved with formalin; the second is good for cells with chloroplasts, such as cultured, autotrophic species, as they have auto-fluorescence.

(a) *Simple method*

This method is useful for samples with a low number of dinoflagellates. Place a droplet of water sample on a glass slide, and carefully place a cover slip over the droplet. Add a droplet of Cellufluor solution along a margin of the cover slip. At the same time, draw excess water from the opposite margin with a paper towel to help the dispersion of the fluorescent stain. Let the slide stand for a few minutes. Next, wash by adding a droplet of distilled water along the cover slip and drawing through as previously described. Observe under ultraviolet light on a fluorescence microscope (excitation 340–400 nm; emission 400–440 nm). The thecal plates will appear pale blue. Prolonged exposure to strong UV irradiation will reduce the fluorescence of the sample.

(b) *Chlorophyll extraction method*

The fluorescence produced will vary depending on the thickness of the thecal plates. Plates of cultured cells are usually thin and weak. Moreover, cultured cells of autotrophic species have strong red auto-fluorescence even after fixation. Auto-fluorescence disturbs blue fluorescence from thecal plates, so the previous method is not applicable to cultured samples, and auto-fluorescence should be removed. Prior to the stain application, chlorophyll should be extracted using methanol.

Pipette cells into a 15 ml centrifuge tube, and fix with 1% glutaraldehyde solution for 30 min. After fixation, collect the fixed cells by a few minutes of centrifugation, and discard the supernatant. Pour cold methanol into the tube with the cell-pellet and place the tube in a refrigerator for one night. Then replace the methanol with 10 ml distilled water using centrifugation. Add about 100 µl of Cellufluor staining solution to the tube, and leave for 30 min in the dark. After staining, remove the supernatant containing Cellufluor solution using centrifugation. Re-suspend the pellet in distilled water, centrifuge again, and remove supernatant to wash. Finally, re-suspend the pellet in distilled water for observation. Observe under fluorescence microscope as in the previous method.

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16

Taxonomy of toxic haptophytes (prymnesiophytes)

Ø. Moestrup and H. A. Thomsen

16.1 Introduction

Haptophyceae was first recognized as a separate class of algae by Christensen (1962), following detailed studies by I. Manton and M. Parke in England. The 'third flagellum', one of the most characteristic features of many haptophyte cells, was shown by Manton and Parke to represent an entirely new organelle, which Parke *et al*. (1955) named the haptonema. Pigments of haptophytes are very similar to chrysophytes, and haptophytes were previously included in the Chrysophyceae, which differ markedly, however, in the heterokont flagellation (a hairy anterior and a smooth posterior flagellum) while haptophytes usually have two equal (isokont) or subequal (anisokont) flagella. More recently, gene-sequencing studies have indicated that the Haptophyceae and the Chrysophyceae are only distantly related (e.g. Leipe *et al*., 1994). Much confusion arose as a result of the recommendation by the *International Code of Botanical Nomenclature* that names above the level of genera should be typified, i.e. based on a generic name. This led Hibberd (1976) to introduce the name Prymnesiophyceae as an alternative but typified class name (based on the genus *Prymnesium)* to replace Haptophyceae. The idea of typified names has not been generally accepted, however, and currently both names Haptophyceae and Prymnesiophyceae are equally valid. It is possible and likely that the classification used in the future will comprise *two* classes rather than one, united as the phylum Haptophyta (a name validated by Edvardsen *et al*., 2000, colloquial name haptophytes), and known as Prymnesiophyceae (prymnesiophyceans) and Pavlovophyceae (pavlovophyceans), the latter differing so markedly in cell structure that separation at the class level appears justified (Green and Leadbeater, 1994). The difference is confirmed by studies of LSU rDNA (Edvardsen *et al*., 2000).

All known toxic haptophytes belong to the Prymnesiophyceae. Currently only species of the genera *Prymnesium* and *Chrysochromulina* are recognized as potentially toxic, but recent findings from Norway indicate that *Phaeocystis*, a cosmopolitan genus of foam-producing species, may also be a toxin producer (Eilertsen and Raa, 1995). The future will undoubtedly demonstrate toxin production in more genera (the coccolithophorid *Ochrosphaera napolitana* was claimed by Jebram (1980) to be toxic) but in the absence of conclusive evidence, the present account deals only with *Chrysochromulina*, *Prymnesium* and *Phaeocystis*.

16.2 Description of the class Haptophyceae

Haptophytes are generally unicellular or colony-forming flagellates, which are particularly common in marine plankton. Most species are small and belong in the nanoplankton, i.e. the cells are usually smaller than 20 µm. They may be recognized as haptophytes by the yellow colour of the (usually two parietal) chloroplasts, the green colour of chlorophylls *a* and *c* being masked by the carotenoid fucoxanthin, which is present in large amounts and photosynthetically active (Jeffrey, 1980). The cells contain a single nucleus that is difficult to see except under high-resolution light microscopes, using Nomarski interference contrast, while a relatively large vacuole in the posterior end is commonly visible. It contains chrysose (chrysolaminarin, leucosin), a water-soluble polysaccharide. The same colour and the same kind of vacuole occurs in the Chrysophyceae, from which haptophytes may be distinguished by their flagellation. The hairy front flagellum of chrysophytes beats in a sine wave as in other heterokonts, pulling the cell forward in the water or, in sessile forms, drawing water towards the cell. The smooth flagellum is often trailing. In prymnesiophyceans both flagella are usually active in swimming, the ventral part being directed anteriorly, while the tip is bent backwards along and behind the cell. Two species of *Chrysochromuliuna* are quadriflagellate (*C. birgeri* and *C. quadrikonta)* while other prymnesiophyceans possess only two flagella. Cells lacking flagella cannot be distinguished from chrysophytes by light microscopy. The marine plankton comprises only a few nano-sized chrysophytes, however, and in the marine nanoplankton small flagellar-less unicellular algae with two parietal yellow chloroplasts are usually haptophytes which have lost flagella and haptonema. Some species lack a haptonema altogether (e.g. *Dicrateria*: Green and Pienaar, 1977) or this may be present as an internal remnant not visible by light microscopy (e.g. *Imantonia*: Green and Pienaar, 1977). All species of *Chrysochromulina, Prymnesium* and *Phaeocystis* possess a haptonema (in *Phaeocystis* only in the flagellate stage, not in the colony stage). In *Phaeocystis* the haptonema is very short and often difficult to see under the light microscope while in *Prymnesium* and particularly in *Chrysochromulina* it is readily visible and in *C. camella* may attain a length of 160 mm (Leadbeater, 1972).

Short haptonemata may be used as attachment organelles (*Prymnesium*) while longer haptonemata may serve in food uptake (Kawachi *et al*., 1991): an increasing number of haptophytes is now being discovered to be mixotrophic (Jones *et al*., 1994). In *Chrysochromulina hirta* the feeding process begins when small eukaryotic algae attach to the haptonema. They are subsequently concentrated in a small packet at the haptonema tip and the haptonema then bends to deliver the packet at the posterior end of the cell (Kawachi *et al*., 1991). The food is ingested into a food vacuole. In *Chrysochromulina ericina*, whose cells are covered with long spines, the prey is caught on the spines and transported to the haptonema, before being delivered at the posterior end of the cell for phagocytosis (Kawachi and Inouye, 1995). A third food-uptake mechanism, which does not involve the haptonema, has recently been found in *Prymnesium patelliferum* (Tillmann, 1998). *Prymnesium* produces an elongate U-shaped pseudopodium from the posterior end of the cell that is used in food uptake, many cells of *Prymnesium* sometimes feeding on the same prey organism (e.g. dinoflagellate, green alga, etc.).

During slow forward swimming, the haptonema is generally directed forwards while the flagella beat slowly backwards along the cell. On hitting an object, a long

haptonema instantly rolls up and swimming ceases abruptly. Slow swimming recommences when the haptonema extends again. Short bursts of rapid swimming occur with all appendages directed backwards behind the cell. *Balaniger balticus* and several coccolithophorids are entirely apoplastidic (Marchant and Thomsen, 1994) but whether these species are phagotrophic or osmotrophic is not known.

Cells of many haptophytes are covered with one or more layers of scales whose function remains obscure. They are used extensively in taxonomy, but only the larger types are visible with the light microscope. Some scales of the coccolithophorids are calcified (coccoliths) while others are non-calcified (organic). *Prymnesium*, *Chrysochromulina* and *Phaeocystis* produce only organic scales. Identification of most haptophytes relies on scale structure, cell size, and length of the flagella and haptonema, the first feature being the most reliable. In many genera identification to species level requires electron microscopical examination of scale morphology; this applies to nearly all species of *Prymnesium* and to most species of *Chrysochromulina*. Transmission electron microscopy (TEM) of whole mounts is to be preferred (Moestrup and Thomsen, 1980), as the resolution of the scanning electron microscope (SEM) is often insufficient to resolve the minute details of the scales. It should also be borne in mind that many species of *Chrysochromulina* are as yet undescribed, thus few studies have been performed on the genus in tropical and oceanic areas. In *Phaeocystis* the species concept is under revision, and identification to species level remains difficult.

Reproduction of haptophytes is usually by longitudinal fission, some species often dividing once per day (*Chrysochromulina polylepis*, *Prymnesium patelliferum* (e.g. Larsen *et al*., 1993). Sexual reproduction is known in some cases (Billard, 1994), but it is unknown in *Prymnesium*, *Chrysochromulina* and *Phaeocystis*. It is likely to occur, however, since a haplo-diploid life cycle has been suggested for these genera (Vaulot *et al*., 1994; Edvardsen and Vaulot, 1996; Larsen and Edvardsen, 1998). Thick-walled resting stages occur, for example, in *Prymnesium* (Pienaar, 1980). Old cultures of *Chrysochromulina* often contain both coccoid and amoeboid stages and cells with odd numbers of flagella (Parke *et al*., 1955). Whether these are haploid or diploid is unknown.

Many haptophytes are distributed worldwide (e.g. *Emiliania huxleyi*, many species of *Chrysochromulina*, etc. (Marchant and Thomsen, 1994; Thomsen *et al*., 1994). A major effort will be required to study the non-coccolithophorid species of tropical areas where only a few species have been reported (e.g. Manton, 1982, 1983; Manton and Oates, 1983) but preliminary studies have shown (Moestrup, unpublished; Thomsen, unpublished) that they are as common in the tropics as in temperate and polar waters. Around 75 genera and 500 species of haptophytes have been described (Green *et al*., 1990).

Some prymnesiophyceans are well-known fish killers and economic aspects of prymnesiophycean blooms have recently been reviewed (Moestrup, 1994).

16.3 KEY TO GENERA

16.3.1 Description of genera

16.3.1.1 *Chrysochromulina* Lackey 1939. Type species: *C. parva* Lackey 1939

Since our first review (Moestrup and Larsen, 1992), in which only a single species was described as toxic, studies in Norway have demonstrated that one more of the around 50 described species is potentially toxic. In addition, our attention was directed to an article by Jebram (1980), who found several species to be toxic in old cultures, when served as food to the bryozoan *Electra pilosa*. Currently five species of *Chrysochromulina* have been claimed to be potentially toxic (Figs. 16.1A–E) while seven have proved non-toxic when tested to the brine shrimp *Artemia* or the bryozoan *Electra* (see Table 16.1). The table provides an overview of characters used to distinguish individual species of *Chrysochromulina* by light microscopy. The potentially toxic species are described in detail below. Many species require further study, and there are probably as many undescribed as formally described species, i.e. *Chrysochromulina* probably comprises 75–100 species.

16.3.1.2 *Chrysochromulina brevifilum* Parke et Manton 1955

Description: Cells spheroidal-pyriform, the flagellar pole usually flattened, (3.5–) 4–7 (-11.5) µm in diameter (Fig. 16.1A). Two equal, homodynamic flagella, 2.5–3.5 times the cell diameter in length (Birkhead and Pienaar, 1994: (11.3–) 13–17 (-19.5) µm), and the haptonema, (3–) 7.6–9.5 (-14.2) µm in length, are attached at the flattened pole. The haptonema is nearly always extended during swimming, rarely coiled up. It is always shorter than the flagella. Two (rarely one or four) golden-brown parietal chloroplasts are present, each with a pyrenoid which is usually difficult to see except in squashed cells. Oil droplets are scattered throughout the cell, and muciferous bodies line the plasmalemma. A vacuole of variable size is sometimes visible posteriorly (phagotrophy?; see below). Cells may become greenish with age.

Scale structure: In the original description only a single type of scale was seen, described as ellipsoid, *c.* 0.7 µm across, each scale with a central spine attached by four decurrent ridges (Parke *et al.*, 1955). Later, Manton (in Leadbeater, 1972, p. 76) mentioned the presence of plate scales and recently Birkhead and Pienaar (1994) found a second type of plate scale in material from South Africa. The presence of three types of scale has been confirmed in a clone from Denmark (Moestrup, unpublished) and Figs. 16.2A, 2B show the two main types of scale. The large plate scales measure 0.76–1.02 µm in length and 0.68–0.92 µm in width. They carry a rim, which is 0.07–0.1 µm wide and shows a pattern of concentric fibres. Each scale is divided into four quadrants, each with about 17 radiating lines visible on both sides of the scale. The spine scales, which occur in numbers similar to the large plate scales, are slightly longer than wide or almost isodiametric, 0.63–0.98 µm long and 0.55–0.84 µm wide. The central spine is 0.6–0.7 µm long with four decurrent ridges that do not extend to the rim. The scales carry an unstructured rim measuring *c.* 0.08 µm. Like the plate scale, the spine scale is divided into four quadrants. These are delineated by the decurrent ridges of the spine, each quadrant with about 15 radiating ridges. The small plate scales are much less numerous (Birkhead and Pienaar found four times as many of the large type of plate scale). They are

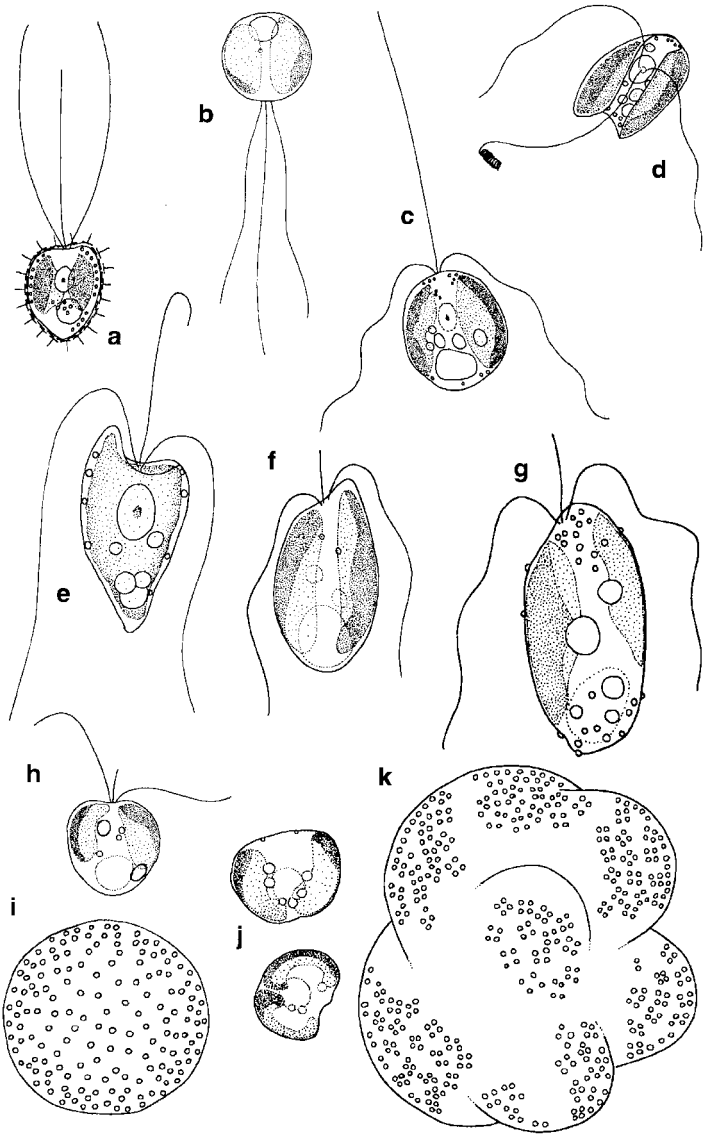

Figure 16.1
Potentially harmful species of Haptophyta. A, *Chrysochromulina brevifilum*; B, *C. leadbeateri*; C, *C. kappa*; D, *C. camella*; E, *C. polylepis*; F, *Prymnesium patelliferum*; G, *P. annuliferum*; H, zooid of *Phaeocystis*; I, colony of *P. globosa*; J, single cells of *Phaeocystis*; K, colony of *P. pouchetii*. All magnifications × 3,000. *Sources:* A, C, redrawn from Parke *et al*. (1955); D, redrawn from Leadbeater and Manton (1969*a*); E, redrawn from Manton and Parke (1962); B, F, H–K, originals, Moestrup and Thomsen; G, redrawn from Billard (1983).

TABLE 16.1 List of *Chrysochromulina* species described, including dimensions and shape of cell body, dimensions of flagella and haptonema, and an indication of habitat, phagotrophy and toxicity*

Species	Hab.	Length			Width			L/W	Shape	Flagella			Haptonema			Hapt./ Flag.	Hapt./ C.L.	Sp. Vis.	Sp. Pos.
		min.	ave.	max.	min.	ave.	max.			min.	ave.	max.	min.	ave.	max.				
C. acantha	m	6	8	10	6	8	10	1,0	saddle		20			40	10	2,0	5,0	n	
C. adriatica	m	5	7	8	6	8	9	0,9	subspherical	8	9	10	6	8	74	0,9	1,1	n	
C. ahrengotii	m	4	5	7	4	5	6	1,1	saddle	12	16,5	21	54	64		3,9	12,2	n	
C. alifera	m	6	8	10	6	8	10	1,0	saddle	12	18	25	60	90	120	5,0	11,3	n	
C. apheles	m	3	4	4	3	5	6	0,8		7	11	15	20	30	40	2,7	7,5	n	
C. bergenensis	m		7		4	4	6	1,8			10			8		0,8	1,1	n	
C. bergenensis (type)	m		4			4		1,0	ovate		10			8		0,8	2,0	n	
C. birgeri	m	13	18	31	8	10	11	1,8		30	33	35	10	15	18	0,5	0,8	n	
C. brachycylindra	m	4	5	7	4	5	7	1,0	subspherical	8	11	13	8	11	13	1,0	2,2	n	
C. brevifilum	m	4	6	12	4	6	12	1,0	spheroid	9	17	25	8	13	18	0,8	2,2	n	
C. camella	m		14			16		0,9	saddle		25		160	160	160	6,4	11,4	n	
C. campanulifera	m		10			10		1,0	saddle		25		40	50	60	2,0	5,0	n	
C. Chiton	m	4	7	10	4	7	10	1,0	spheroid	13	20	28	2 20	30	40	1,5	4,3	n	
C. cyathophora	m		3			3		1,0	subspherical	10	11	12	0	7		0,6	2,3	n	
C. cymbium	m		7			7		1,0	saddle		20			60		3,0	8,6	n	
C. discophora	m		10			10		1,0	spherical		25		25	30		1,2	3,0	y	
C. elegans	m	3	4	4	3	4	4	1,0		14	16	17		25		1,6	6,3	y	
C. ephippium	m	6	8	10	6	8	10	1,0	saddle	18	29	40	72	106	140	3,7	13,3	n	
C. ericina	m	5	8	12	4	7	10	1,1	ovoid	12	18	25	24	37	50	2,1	4,6	y	all
C. 'eyelash'	m	10	11	12	10	11	12	1,0	saddle	20	22	23	6	7,5	9	0,3	0,7	n	
C. fragaria	m	4	6	4	4	6	8	1,0	spherical	10	13	16	3	6	9	0,5	1,0	n	
C. fragilis	m		4			4		1,0	subshperical	10	13	15		7,5		0,6	1,9	n	
C. fragilis aff.	m		6			5		1,2			13			7,5		0,6	1,3	n	
C. herdlensis	m	6	7	7	5	6	7	1,2			35			15		0,4	2,1	n	
C. herdlensis (type)	m		4			3		1,3	conical		35			15		0,4	3,8	n	
C. hirta	m		6	12		6	12	1,0			20			30		1,5	5,0	y	all
C. kappa	m	4	6	10	4	6	10	1,0	spheroidal	7	11	16		14		1,3	2,3	n	
C. latilepis	m		9			6		1,5	ovoid	10	15	20		25		1,7	2,8	y	
C. leadbeateri	m	3	6	8	3	6	8	1,0		13	17	20	20	22	24	1,3	3,7	n	
C. leadbeateri (type)	m	2	3	4	2	3	4	1,0		11	12	13		15		1,3	5,0	n	

Species	Hab.	Length			Width			L/W	Shape	Flagella			Haptonema			Hapt./ Flag.	Hapt./ C.L.	Sp. Vis.	Sp. Pos.
		min.	ave.	max.	min.	ave.	max.			min.	ave.	max.	min.	ave.	max.				
C. mactra	m	15	18	20	15	18	20	1,0		20	30	40	30	35	40	1,2	1,9	n	
C. mantoniae	m		10		4	5	5	2,0			19			10		0,5	1,0	y	
C. mantoniae (type)	m		6			3		2,0	subcylindrical	18	19	20		10		0,5	1,7	y	polar
C. megacylindra	m		5			4		1,3	conical		20			20		1,0	4,0	n	
C. microcylindra	m	4	6	7	4	6	7	1,0	subspherical	20	23	25	16	23	30	1,0	3,8	n	
C. minor	m	3	4	8	3	4	8	1,0	spheriod	6	11	15		9		0,8	2,3	n	
C. novae-zelandiae	m		7			7		1,0			25			5		0,2	0,7	n	
C. orbiculata	m	4	6	7	4	6	7	1,0	spheroid	7	11	15	5	8,6	12	0,8	1,4	n	
C. pachycylindra	m		8			8		1,0	globose		20			15		0,8	1,9	y	all
C. parkeae	m	10	20	30	5	8	10	2,5	elongate	8	20	30	2,5	5	10	0,3	0,3	y	polar
C. pelagica	m	6	8	10	6	8	10	1,0		22	24	25		35		1,5	4,4	y	
C. polylepis	m	6	9	12	5	7	9	1,3	ovoid	18	23	27	9	11	13	0,5	1,2	n	
C. pontica	m	4	5	5	4	5	5	1,0		8	9	10		70		7,8	14,0	n	
C. pringsheimii	m	12	17	24	4	7	9	2,4	cylindrical	21	30	40	14	27	40	0,9	1,6	y	polar
C. pyramidosa	m		3			4		0,8		10	12	13	5	5,5	6	0,5	1,8	n	
C. quadrikonta	m	10	18	25	10	14	18	1,3	subspherical	30	35	40	25	28	30	0,8	1,6	y	all
C. rotalis	m	4	5	6	4	5	6	1,0	saddle	8,	11	14	22	38,5	55	3,5	7,7	n	
C. scutellum	m	4	7	9	4	5	8	1,3	saddle	12	16	20	13	46,5	80	2,9	7,2	n	
C. simplex	m		6			5		1,2		8	14	20		78	78	5,6	13,0	n	
C. simplex (type)	m	2	4	6	2	4	6	1,0	saddle	9	18	27		50		2,8	12,5	n	
C. spinifera	m	8	9	10	7	8	9	1,1		7	9	12	4	4,5	5	0,5	0,5	y	all
C. spinifera (type)	m	6	7	8	3	4	5	1,8	bell	10	20	30	6	7	8	0,4	1,0	y	polar
C. strobilus	m	6	8	10	6	8	10	1,0	saddle	12	21	30	72	126	180	6,0	15,8	n	
C. tenuispina	m	8	11	13	8	11	13	1,0	globose		25	30		20	25	0,8	1,8	y	all
C. tenuisquama	m	2	4	5	2	4	5	1,0		7	13	19	39	45	52	3,5	11,3	n	
C. throndsenii	m	5	6	6	5	6	6	1,0	saddle	12	12	12	32	41	50	3,4	7,5	n	
C. vexillifera	m		8			6		1,3			20		20	25		1,3	3,1	y	polar
C. breviturrita	f	6	10	16	6	10	16	1,0	spheroidal	15	22	27	6	12	16	0,5	1,2	n	
C. inornamenta	f	6	8	10	6	8	10	1,0	spherical-ovate	18	21	24	8	11	14	0,5	1,4	n	
C. laurentiana	f	7	8	9	7	8	9	1,0	spheroid	18	19	20	10	13	15	0,7	1,6	n	
C. parva	f	3	5	7	3	5	7	1,0			15			75		5,0	15,0	n	

* Hab., habitat; C.L. cell length; Vis., visible; Pos., position on cell (all sides or polar); Ph., phagotrophy.

…/…

TABLE 16.1 *(Suite)*

Species	Comments	Ph.	Toxic	References
C. acantha		y	n	Leadbeater and Manton (1971)
C. adriatica				Leadbeater (1974)
C. ahrengotii			n	Jensen and Moestrup (1999)
C. alifera		y		Parke *et al.* (1956)
C. apheles			n	Moestrup and Thomsen (1986)
C. bergenensis		y		Manton and Leadbeater (1974)
C. bergenensis (type)		y		Leadbeater (1972)
C. birgeri				Hällfors and Niemi (1974); Hällfors and Thomsen (1979)
C. brachycylindra				Hällfors and Thomsen (1985)
C. brevifilum		y	y, n	Parke *et al.* (1955)
C. camella		y	n	Leadbeater and Manton (1969*a*)
C. campanulifera	Haptonema substantially longer than flagella	y		Manton and Leadbeater (1974)
C. Chiton		y		Parke *et al.* (1958)
C. cyathophora				Thomsen (1979); Manton *et al.* (1981)
C. cymbium		y		Leadbeater and Manton (1969*a*)
C. discophora	Haptonema>25 µm			Manton (1983)
C. elegans	Haptonema longer than flagella			Estep *et al.* (1984)
C. ephippium		y		Parke *et al.* (1956); Manton and Leadbeater (1974)
C. ericina			n	Parke *et al.* (1956); Leadbeater (1972)
C. 'eyelash'	Haptonema non-coiling	y		Pienaar and Bandu (1984)
C. fragaria			n	Eikrem and Edbardsen (1999)
C. fragilis				Leadbeater (1972)
C. fragilis aff.				Manton and Leadbeater (1974)
C. herdlensis		y		Manton and Leadbeater (1974)
C. herdlensis (type)		y		Leadbeater (1972)
C. hirta	Haptonema somewhat longer when extended	y	n	Manton (1978); Kawachi *et al.* (1991)
C. kappa	Haptonema a little longer than flagella	y	y, n	Parke *et al.* (1955)
C. latilepis	Haptonema long; >flagella			Manton (1982)
C. leadbeateri	Flagella slightly anisokont		y, n	Eikrem and Throndsen (1998)
C. leadbeateri (type)	Haptonema longer than flagella			Estep *et al.* (1984)

.../...

Species	Comments	Ph.	Toxic	References
C. mactra	Plate scales perhaps visible			Manton (1972)
C. mantoniae	Haptonema non-coiling			Manton and Leadbeater (1974)
C. mantoniae (type)	Haptonema non-coiling			Leadbeater (1972)
C. megacylindra		y		Leadbeater (1972); Manton (1972)
C. microcylindra				Leadbeater (1972)
C. minor	Haptonema slightly shorter than flagella	y	n	Parke *et al.* (1955)
C. novae-zelandiae				Moestrup (1979)
C. orbiculata				Rouchijajnen (1972)
C. pachycylindra	Haptonema shorter than flagella; flagella >20			Manton *et al.* (1981)
C. parkeae	Haptonema non-coiling			Green and Leadbeater (1972)
C. pelagica	Haptonema longer than flagella			Estep *et al.* (1984)
C. polylepis		y	y, n	Manton and Parke (1962); Edvardsen and Paasche (1992)
C. pontica				Rouchijajnen (1966)
C. pringsheimii		y	n	Parke and Manton (1962)
C. pyramidosa				Thomsen (1977)
C. quadrikonta	Four flagella; proximate part of haptonema covered by spine scales			Kawachi and Inouye (1993)
C. rotalis				Eikrem and Throndsen (1999)
C. scutellum			n	Eikrem and Moestrup (1998)
C. simplex			n	Birkhead and Pienaar (1990)
C. simplex (type)	Haptonema much longer than flagella			Estep *et al.* (1984)
C. spinifera	Haptonema non-coiling	y		Pienaar and Norris (1979)
C. spinifera (type)	Haptonema non-coiling	y		Fournier (1971)
C. strobilus		y	y(?)	Parke *et al.* (1959); Leadbeater and Manton (1969*b*)
C. tenuispina	Haptonema not much shorter than flagella			Manton (1978); Kawachi *et al.* (1991)
C. tenuisquama				Estep *et al.* (1984)
C. throndsenii			n	Eikrem (1996)
C. vexillifera	Haptonema >20 µm			Manton and Oates (1983)
C. breviturrita				Nicholls (1978)
C. inornamenta				Wujek and Gardiner (1985)
C. laurentiana				Kling (1981)
C. parva			n	Lackey (1939)

0.57–0.71 µm long and 0.52–0.66 µm wide and basically similar to the large plate scales. Each scale has a rim, *c*. 0.08 µm wide, with a concentric pattern. The scales are divided into four quadrants, each quadrant with 12–13 radiating ridges that are visible from both sides of the scales. None of the three scale types is confined to any particular region of the cell.

Ecology and distribution: *C. brevifilum* was described from the sea near Plymouth (Parke *et al*., 1955) and has subsequently been found in Denmark (Manton and Leadbeater, 1974; Jensen, 1998), Norway (Leadbeater, 1972; Eikrem, pers. comm.) and South Africa (Birkhead and Pienaar, 1994). It is probably very widely distributed but usually has not been identified to species level. The micrographs showing *C. brevifilum* in Australia (Hallegraeff, 1983, Fig. 7) and New Zealand (Moestrup, 1979, as aff. *C. brevifilum*, Figs. 8, 9) almost certainly belong to other species. The scales of the former are too small and with too few radiating ridges (about 36) while the latter organism has two types of scale, smaller spineless plate scales and slightly larger spine-carrying scales.

Toxicology: Jebram (1980) found old cultures of *C. brevifilum* to be toxic to the bryozoan *Electra pilosa*. Simonsen and Moestrup (1997) found no toxicity of *C. brevifilum* to the brine shrimp *Artemia salina*, neither in complete nor in phosphorus-deplete medium.

Note: *C. brevifilum* is phagotrophic and ingests bacteria or graphite particles up to a size of 2 µm or 2.5 µm (Parke *et al*., 1955; Jones *et al*., 1993).

16.3.1.3 *Chrysochromulina kappa* Parke et Manton 1955

Description: Cells spheroidal (Fig. 16.1C), somewhat metabolic, (4–) 4.5–6.5 (-10.5) µm in diameter. The two flagella and the haptonema arise close together at one pole. The flagella are equal, homodynamic, the length 1.5–2.5 times the cell diameter. The haptonema is slightly longer than the flagella. The cell contains two or four golden-brown chloroplasts (rarely one or none). Each chloroplast has a bulging pyrenoid (Manton and Leedale, 1961; Moestrup and Eikrem, in preparation).

Scale structure: The original rather meagre description states that the cell is covered with ‘very thin transparent sculptured scales, on any one cell ranging in shape from oval to polygonal and in size from 0.3×0.4 µm to 0.5×0.8 µm with, at

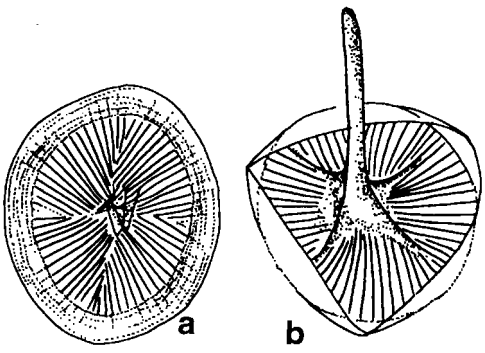

Figure 16.2 A, B
Scales of *Chrysochromulina brevifilum*.
Source: originals, from Danish material.

the flagellar pole, a few scales bearing a soft central spine' (Parke *et al*., 1955). Based on new isolates established from Norway (Moestrup and Eikrem, in preparation) we know that this species has three types of scale (Figs. 16.3A–C): plate scales of two sizes cover the cell throughout (Figs. 16.3A, B), while the spine scales (Fig. 16.3C) are confined to the flagellar pole. All scale types have a radiating pattern of microfibrils on the ventral scale face and a pattern of crossed microfibrils on the dorsal scale face. The largest plate scales are ovoid, the smallest almost round. The largest scales measure 0.6–0.7 × 0.5–0.6 µm, the smallest 0.3–0.4 µm in diameter. The largest scales have a raised rim on the dorsal side, the small scales a narrow inflexed rim. Both scale types show a pattern of radiating lines arranged in quadrants. The small scales have 9–10 lines in each quadrant, the larger scales 14–15 lines. The spine scales are few in number and circular. They measure *c.* 0.5–0.6 µm in diameter. The scales are thin and the radiating fibres on the ventral side are visible also when scales are seen in dorsal view. A low, raised rim is also present. The central spine is shorter than the radius of the scale or equal to it. It is attached by four decurrent ridges that extend to the rim of the scale, or nearly so.

Ecology and distribution: *C. kappa* was described from the Isle of Man and the south coast of England (Parke *et al*., 1955). Leadbeater (1972) and Eikrem (in Edvardsen *et al*., 2000) subsequently found it on the west coast of Norway. Finds from other geographical areas, confirmed by illustrations of scale structure, have apparently not been made.

Toxicology: Old cultures of *C. kappa* were found by Jebram (1980) to be toxic to the bryozoan *Electra pilosa*. Edvardsen (1993) found it non-toxic to the brine shrimp *Artemia*, in both complete and phosphorus-deplete medium.

Note: *C. kappa* is phagotrophic, ingesting particles up to 2.5 µm in diameter, including bacteria (Parke *et al*., 1955).

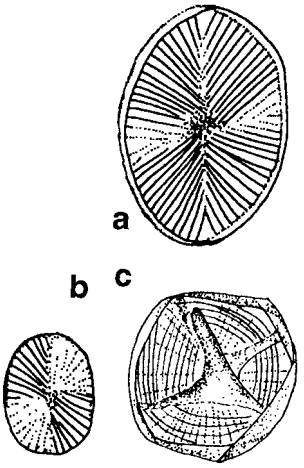

Figure 16.3 A–C
The three scale types of *Chrysochromulina kappa*.
Source: originals, Moestrup and Thomsen.

16.3.1.4 *Chrysochromulina leadbeateri* Estep, Davis, Hargraves et Sieburth 1984

Description: Cells more or less spherical (Fig. 16.1B), 3–8 µm in diameter. The two flagella are 13–16 µm and 16–20 µm long, the haptonema *c.* 20 µm long. All appendages are attached anteriorly but the haptonema is usually coiled up. Cells contain two golden-brown parietal chloroplasts, each with a pyrenoid. (This description is based on material from Lofoten, Norway, by Eikrem and Throndsen, 1998).

Scale structure: The cell surface is covered with two types of scale, arranged in two layers (Figs. 16.4A, B), each with a circular outline, and measuring *c.* 0.35 µm in diameter. Both scale types possess a central area with a single cross. Scales of the underlayer are plate-like and rimless, consisting of concentric and radiating ribs (Fig. 16.4A). The underlayer shows a regular pattern of about 25 radiating and three concentric ribs, the innermost ring encircling the central cruciate structure. The second concentric rib divides the scale into an intermediate and a dorsal part, with single perforations between all radiating and concentric ribs. The other scale type (Fig. 16.4B) is less ornamented but surrounded by a shallow upright rim. The only pattern is the central cross, the rest of the scale appears structureless (description based on material from the Atlantic Ocean drawn by Eikrem and Throndsen, 1998).

Ecology and distribution: Material identified as *C. leadbeateri* has been reported from many parts of the world. The species concept is, however, somewhat uncertain due to the finding of slightly different scale morphotypes in different parts of the world (Eikrem and Throndsen, 1998). These differences apply to both types of scale, thus the material from Lofoten responsible for the fish killings is slightly different from the type material of *C. leadbeateri* collected in the Atlantic during a cruise from West Africa to the Caribbean (Estep *et al*., 1984). A reliable species concept awaits establishment of the clones in culture for detailed examinations, including studies of gene sequences.

Toxicology: *C. leadbeateri* was responsible for killing 600 tons of cultured fish in northern Norway (Lofoten) in 1991 (Throndsen and Eikrem, 1991). The toxic principle has not been isolated. Cultures of *C. leadbeateri*, however, proved non-toxic to the brine shrimp *Artemia salina* (Edvardsen, 1993; Meldahl *et al*., 1994; Simonsen and Moestrup, 1997), and three other methods also failed to demonstrate any toxicity.

16.3.1.5 *Chrysochromulina polylepis* Manton et Parke 1962

Description: Cells of variable shape, usually ovoid or spherical (Fig. 16.1E), the flagellar pole obliquely truncate and depressed to form a groove. Cells 6–12 µm long and 5–9 µm wide. Flagella homodynamic, equal or subequal in length, 2 to 3 times

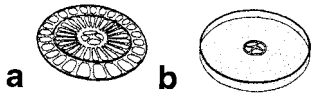

Figure 16.4 A–B
Scales of *Chrysochromulina leadbeateri* (Atlantic Ocean).
Source: Eikrem and Throndsen (1998).

cell length, haptonema 1.1–5 times cell length, coiling, inserted subapically. The haptonema is capable of attaching with its swollen tip. Two (rarely four) golden-brown parietal chloroplasts.

Scale structure: Cells covered with four types of scale (typical form; aberrant form, see below). The entire cell is covered with two types of scale, arranged in two layers. Scales of the inner layer (Fig. 16.6A) are oval, measuring 0.7–0.8 µm × 0.5–0.6 µm. They possess an inflexed patternless rim on the dorsal surface, *c.* 0.08 µm in width. The number of perforations in the (usually) six concentric rings increases from about four near the centre to about 50 along the periphery. Perforations of the outermost ring are distinctly elongate, while other perforations are circular or slightly oval. A central thickening separates the four perforations near the centre. These scales are overlaid by larger oval scales (Fig. 16.6B), measuring 1.3–1.4 µm × *c.* 0.8 µm in Danish material. Somewhat wider or even almost isodiametrical scales were seen by Manton and Parke (1962) who gave the dimensions as 1.4 µm × 1.2 µm. These scales possess an upright patternless rim, 0.06–0.07 µm high. Perforations are more numerous than in the underlayer scales. They are more distinctly arranged in four quadrants and in 9–10 rings. The scale in Fig. 16.6B has 85 perforations in the outermost circle, gradually decreasing in number towards the scale centre. A central cross-like thickening is present. The two remaining scale types occur only near the flagellar pole. One type carries a short spine (Fig. 16.6C) while the other, more numerous, scale type, lacks a spine (Fig. 16.6D). Only very few spine scales are present on each cell, thus four were illustrated by Edvardsen and Paasche (1992). The few scales found in Danish material were *c.* 2.5 µm long (as in the type material from England), excluding the rim but including the 0.2–0.3 µm long spine, which bifurcated distally. The width was *c.* 0.5 µm, excluding the patternless rim, which measured *c.* 0.07–0.09 µm in width (width given as *c.* 0.9 µm by Manton and Parke, 1962). In Norwegian material, Eikrem (pers. comm.) sometimes found scales with a spine extending from each end of the scale, and each spine was sometimes with more than one branching. This scale type also shows perforations, but most are filled in and not very conspicuous. The last scale type was seen repeatedly in thin sections of the Danish material (Throndsen *et al*., 1995), providing additional information not available to Manton and Parke (1962). The scales usually measure *c.* 1.0 × 0.5 µm in the Danish material (1.2 × 0.6 µm given as average in the English material) and surrounded by a *c.* 0.04 µm high upright rim a short distance from the scale periphery. Perforations are less numerous than in the two main types of scale, but are arranged in a radiating pattern.

Life cycle: In cultures of Norwegian material Paasche *et al*. (1990) reported the existence of cells with a different scale complement. Only three types of scale were

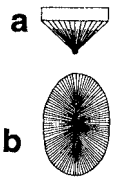

Figure 16.5 A–B
Chrysochromulina camella. Magnification *c.* × 40,000.
Source: originals, Moestrup and Thomsen.

present, one carrying a distinct central spine with four decurrent ridges (Fig. 16.7A). The other scale types (Figs. 16.7B, C) resembled the main scales of the type, but the largest type of scale (Fig. 16.7B) was found to be about 30% longer and wider than in the type. The scales were described as thicker than in the type, and the pores, rather than being in the form of small holes, appeared as 'small irregular openings in a mesh created by radiating and concentric elements (fibrils)' (Paasche *et al*., 1990). Using ploidy and gene analysis it was concluded that the aberrant stage was part of the life cycle of *C. polylepis* (Edvardsen and Vaulot, 1996; Edvardsen and Medlin, 1998). Typical cells were always haploid while aberrant cells were either haploid or diploid, and *C. polylepis* is therefore thought to have a haplo-diploid life cycle. Scales of the aberrant stage have also been found in nature (Edvardsen and Medlin, 1998; Moestrup, unpublished observations).

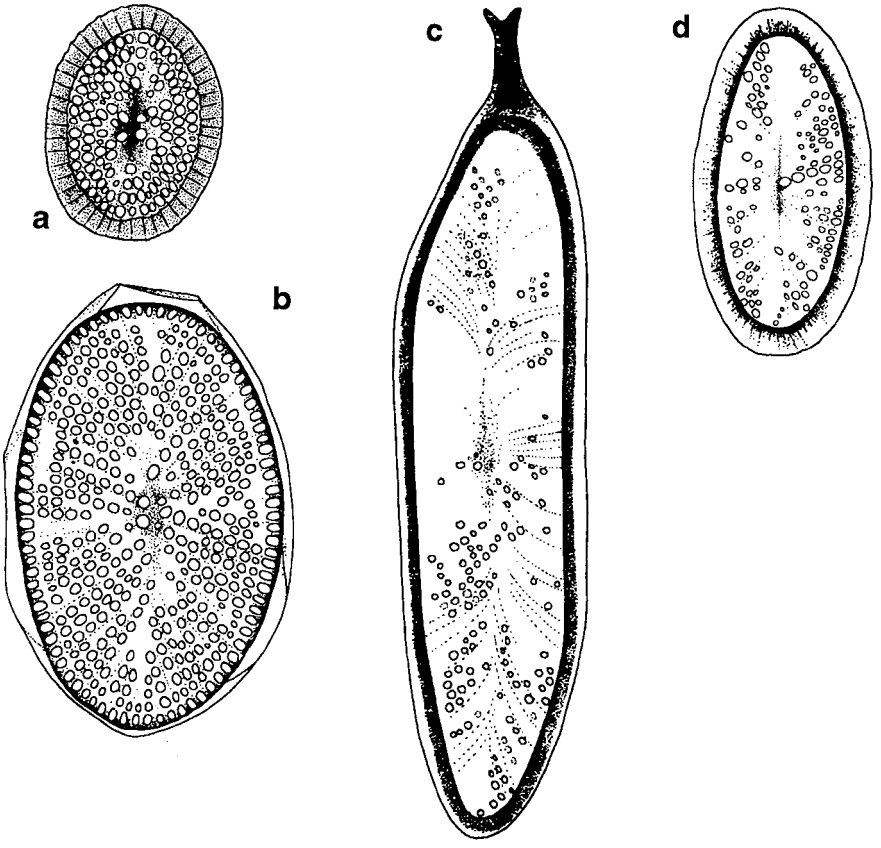

Figure 16.6
Scales of *Chrysochromulina polylepis*. A, B, the most numerous types, which cover the cell body throughout; C, D, the two scale types restricted to the anterior end of the cell.
Source: originals, Moestrup and Thomsen.

Ecology and distribution: *C. polylepis* is currently known from the Irish Sea (Manton and Parke, 1962), Norway, Sweden and Denmark (Barth and Nielsen, 1989), and Australia (Beech and Hill, pers. comm.). Arlstad (1991) found the species to grow equally well at salinities from 10–30‰, and slightly more slowly at 5‰ and 35‰ (in all cases around one division per day under the light conditions used). During the massive occurrence of *Chrysochromulina* in Scandinavian waters in 1988 the cells in some areas were confined to the pycnocline, occurring as a band at several metres depth (Nielsen *et al*., 1990). Bacterivory was recently reported by Nygaard and Tobiesen (1993).

Toxicology: *C. polylepis* was responsible for the massive damage to the environment in 1988 in Scandinavia. Some 900 tons of fish died in Sweden and Norway (mainly cod, salmon and trout), and *Chrysochromulina* also affected invertebrates,

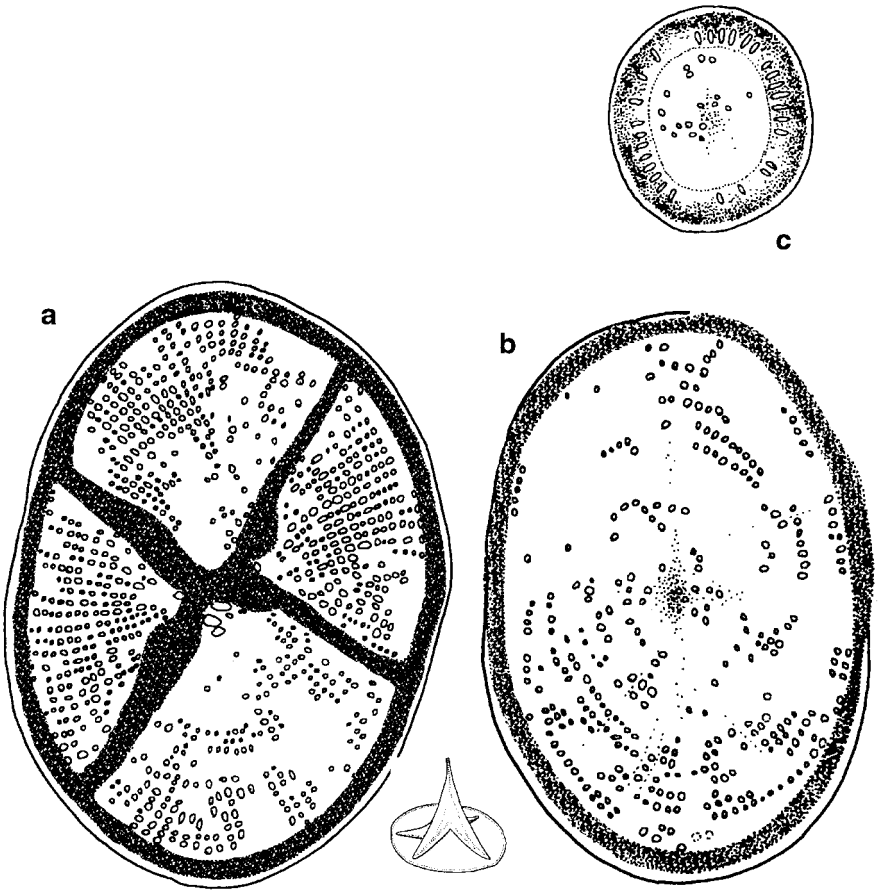

Figure 16.7 A–C
Scales of the aberrant form of *Chrysochromulina polylepis*.
Magnification × 40,000.
Source: originals, Moestrup and Thomsen.

macroalgae, zoo- and phytoplankton, and bacteria (the latter were absent in the water layer containing the toxic cells). The toxin(s) were examined by Yasumoto *et al*. (1990) who found two hemolytic and ichthyotoxic compounds. The major hemolytic compound was a galactolipid, 1-acyl-3-digalacto-glycerol. Small amounts of a poly-unsaturated fatty acid, octadecapentaenoic acid, was also detected. Both the main type and the aberrant form are toxic.

16.3.1.6 *Chrysochromulina strobilus* Parke et Manton 1959

Description: Cells of variable shape: saddle-shaped, bell-shaped, obovoid or flattened globose (Fig. 16.1D), 5–12 µm in size. The flagella are subequal to equal, homodynamic when cells are moving rapidly, heterodynamic when cells move slowly or are stationary, 2–3 times cell length (average 20 µm). The haptonema is 12–18 times the cell body length (average 100 µm), coiling, the number of gyres in the coiled state 25–45 times. The appendages are inserted ventrally 1/3 of the cell length from the anterior end. Cells with two (rarely four) parietal golden-brown chloroplasts, each with an immersed pyrenoid (Parke *et al*., 1959; Leadbeater and Manton, 1969*b*).

Scale structure: The cells carry an outer layer of mucilage, which may obscure details of the scales. Cells are covered with scales of two types (Figs. 16.5A, B). Scales of the inner layer (Fig. 16.5B) are oval flat plates with a pattern of radiating ridges on both surfaces (44 are visible in Leadbeater and Manton 1969*b*, Fig. 16), the ridges converging to a plain centre and showing some indication of being arranged in four quadrants. The scale margin is slightly thickened. The outer scales (Fig. 16.5A) are cup-shaped with a very short stalk, a cone-shaped mid-piece and an upper rim. The base of the cone and the rim are patternless, but the outer surface of the rest of the cone is delicately striated with a single row of perforations just below the junction with the rim.

Similar species: *C. strobilus* has given its name to the strobilus-complex, a group of (to date) four species which are very closely related and unusual in containing six rather than seven microtubules in the haptonema. They are difficult to distinguish without thin sections of the scales and, because of the very close relationship between the four species, they probably behave in a similar way with regard to toxins. For a description of *C. camella* and *C. cymbium*, see Leadbeater and Manton (1969*a*), for *C. campanulifera*, see Manton and Leadbeater (1974).

Ecology and distribution: *C. strobilus* was described from the open sea near Plymouth (England) and subsequently found in Denmark (Manton and Leadbeater 1974).

Toxicology: Old cultures of *C. strobilus* were reported by Jebram (1980) to be toxic to the bryozoan *Electra pilosa*. Rhodes *et al*. (1994) found a New Zealand isolate of the closely related species *C. camella* to be non-toxic to the brine shrimp *Artemia salina* under all conditions tested. A similar lack of toxicity was found by Edvardsen (1993) in *C.* cf. *campanulifera.*

Note: *C. strobilus* is phagotrophic, ingesting bacteria, diatoms, etc. (Parke *et al*., 1959).

16.3.1.7 The genus *Phaeocystis* Lagerheim 1893. Type species: *P. pouchetii* (Hariot) Lagerheim 1893

Phaeocystis is a genus of foam-producing species distributed worldwide and causing problems for fishing and tourism in areas as widely scattered as New Zealand

(Tasman Bay slime) and the North Sea (review by Davidson and Marchant, 1992). Recent evidence from Norway has shown that in addition to the problems caused by the foam, *Phaeocystis* may also have a toxic effect on cod larvae (Eilertsen and Raa, 1995, Aanesen *et al*., 1998; Stabell *et al*., 1999). *Phaeocystis* usually forms colonies (Figs. 16.1I, K) and the morphology of the individual colonies is highly variable. Several species of *Phaeocystis* were described mainly around the turn of the twentieth century but these 'old' species were all merged into one by Kornmann (1955) and Kashkin (1963), followed by Sournia (1988). This trend has now been reversed, and several authors have suggested that the genus comprises several species for which some of the old names are being applied (see below).

Morphology: At least two different stages occur in the life cycle, a colony-forming (Figs. 16.1I, K) and one or more unicellular stages (Fig. 16.1H). In some species colonies may attain a length of *c*. 1 cm and thus become visible to the naked eye. The cells are embedded in mucilage, forming a mono-layer along the periphery of the colony. Each cell contains two (one to four) parietal yellow-green chloroplasts (Fig. 16.1J) but usually lack flagella and haptonema. The unicellular stages (Fig. 16.1H) are biflagellate, 3–9 µm long, with a very short haptonema that is often very difficult to see with the light microscope. The cells usually contain two chloroplasts. The cell surface is covered with submicroscopic flat scales of two kinds. In some cases, trichocyst-like chitinous structures have been found, appearing as pentagonal or nonagonal stars after discharge from the cell (Parke *et al*., 1971, Chrétiennot-Dinet *et al*., 1997). The life history of *Phaeocystis* is still unresolved, and both the colonies and the single cells may self-replicate, the colonies by fragmentation (for a recent reference on the life cycle, see Peperzak *et al*., 2000). It seems probable that some colonies originate from single cells which resorb the appendages and divide into colonies. Single or few-celled stages attached to the setae of diatoms may represent flagellates that have settled. They may eventually give rise to the free-floating colonies, which can contain thousands of cells. Vaulot *et al*. (1994) in several strains of *Phaeocystis* found two ploidy levels, haploid cells being linked to the flagellated stage and diploid cells to the colony-forming stage.

Taxonomy: The taxonomy of *Phaeocystis* is in a phase of transition. Medlin *et al*. (1994) recognized three species, based on the morphology of the colonies, geographical distribution, growth in relation to temperature and 18S small subunit ribosomal DNA sequencing, in addition to *P. scrobiculata,* which is known only from the flagellated stage and differs markedly from the others. More recently Zingone *et al*. (1999) described a further two species from the Mediterranean, bringing the total number of accepted species to six.

- *P. pouchetii* (Hariot) Lagerheim (Fig. 16.1K) is a cold-water species which occurs in the Arctic and in temperate waters of the northern hemisphere. Colonies are rather small, up to 2 mm. Small colonies are spherical, while colonies larger than 0.3 mm are lobed. Cells are generally in groups of four forming a square. Growth optimum is at 8°C, but the colonies will tolerate from less than -2°C to 14°C.
- *P. antarctica* Karsten is a cold-water species confined to the area around Antarctica. It grows to at least 9 mm(?), and colonies are spherical or derived from a spherical shape. Cells are uniformly distributed along the periphery of the colony. It has a growth optimum at 4.5°C and will tolerate from less than -2°C to 14°C. It is closely related to *P. pouchetii* but the distribution of the two taxa does not overlap.

- *P. cordata* Zingone et Chrétiennot-Dinet is known only as flagellated unicells, not colonies. It was described from the Mediterranean Sea near Naples (Zingone *et al*., 1999). The cells are compressed, cordate, but the most characteristic feature is probably the structure of the largest of the two types of scale which cover the cell. In contrast to other species of *Phaeocystis*, the large scales of *P. cordata* show a central knob, and the scales possess a distinct external rim giving the cell surface a rough appearance in SEM.
- *P. globosa* Scherffel (Fig. 16.1I) occurs in temperate waters of both hemispheres. It grows to 8–9 mm and the colonies are (like *P. antarctica*) spherical or derived from a spherical shape. It also resembles *P. antarctica* in the uniform arrangement of the cells along the periphery. It has a growth optimum of 16°C but will tolerate -0.6°C to 22°C.
- *P. jahnii* Zingone is known only from the Gulf of Naples. The cells form loose aggregates in which they are not arranged in any apparent order. The colonies lack a definite shape and a visible external envelope (Zingone *et al*., 1999). As in the other species, motile cells are covered with two types of scale, but scales of the larger type are rimless, extremely thin, and slightly longer (*c*. 0.35 µm) than in the closely related species *P. pouchetii*, *P. antarctica*, *P. cordata* and *P. globosa*, which measure 0.18–0.27 µm. This species also possesses somewhat anisokont flagella.

Except for *P. jahnii*, all these species are known to form pentagonal trichocyst-like structures and the motile cells are scale-covered, while cells of the colonial stage usually lack scales.

- *P. scrobiculata* Moestrup is known only as unicellular flagellate stage. It has considerably longer flagella (23–30 µm versus 6–15 µm), and the two scale types on the cell are markedly different. Both types show a pattern of radiating ridges on the ventral surface while the dorsal side is patternless. The scales in *P. scrobiculata* are oval and measure 0.6 µm × 0.45 µm, the small scales are circular-oval, measuring 0.19–0.21 µm. The trichocyst-like structures of *P. scrobiculata* are nine-rayed stars, as opposed to five-rayed in the other species. *P. scrobiculata* was originally described from New Zealand (Moestrup, 1979) but has also subsequently been found in Australia (Hallegraeff, 1983), the North Pacific (Hoepffner and Haas, 1990), and in temperate and tropical waters of the Atlantic (Estep *et al*., 1984).

Toxicology: The main effect of *Phaeocystis* is clogging of fishing nets, destroying of fish, and formation of masses of foam, which may reach several metres in thickness (Lancelot *et al*., 1987). The foam appears when blooms are washed ashore. Like many other marine plankton algae, *Phaeocystis* blooms produce dimethylsulphide (DMS), which is believed to evaporate to the atmosphere and contribute to the acidity of rainwater (Davidson and Marchant, 1992). A directly toxic effect is believed to have occurred in Norway in 1992, causing death of farmed salmon valued at NKr1 million (Tangen, pers. comm.). The toxic compound(s) has not been identified.

16.3.1.8 The genus *Prymnesium* Massart ex Conrad 1926. Type species: *P. saltans* Massart ex Conrad 1926

Prymnesium (Figs. 16.1F, G) is one of the most serious fish killers (reviewed by Moestrup, 1994), and it is therefore unfortunate that the species cannot be identified by light microscopy. All described species appear very similar, if not identical, by

light microscopy, and identification to species level is not possible without examination of the submicroscopic scales. The exception is one of the most recently described species, *P. nemamethecum*, in which the scaly periplast also covers the haptonema, and this 'haptonema sheath' is visible in the light microscope (Pienaar and Birkhead, 1994). The genus as such may be distinguished, however. The shape of the cell varies from elongate pyriform to almost spherical, not compressed, 6–18.5 µm long by 3.5–11 µm wide. The front end is obliquely truncate and the appendages are inserted subapically in a slight depression of the cell (Figs. 16.1F, G). The flagella are subequal or unequal, 9.5–25 µm in length and heterodynamic. Green *et al*. (1982) describe the flagellar motion as follows: the more posteriorly inserted flagellum is directed posteriorly often close to the cell body and beats with an undulating motion, while the other flagellum passes over the most anterior part of the cell, backwards along the side opposite to that on which the appendages are inserted and beats with a violent flickering movement. The haptonema is short, 2.2–6.3 µm long and non-coiling. It is kept stiffly in front of the cell. The cell possesses two parietal chloroplasts.

Behaviour: Perhaps the most typical feature of *Prymnesium* is the ability of the cells to attach to other objects. This was noted in some of the very first descriptions (Massart, 1920) and is readily visible. In a microscopic preparation the cells attach to the cover slip by the tip of the haptonema, and the cell may rest in this position for some time. Conrad and Leloup (1938) noted that the cells attached to the gills of fish with the haptonema, which they suggest function as a 'style inoculateur', injecting poison into the gills. Experimental evidence for this interesting claim is lacking, however. During attachment, the flagella beat homo- or heterodynamically along and behind the cell. In nature, the cells have been seen to attach to macroalgae or fishing nets (Johnsen and Lein, 1989), or to sand grains (Pienaar and Birkhead, 1994: *P. nemamethecum*). A thick layer of *Prymnesium* was noted by a fisherman after a salmon net had been in the water for a few hours (Johnsen and Lein, 1989). It is possible that the cells graze bacteria in this condition, *P. parvum* was shown by Nygaard and Tobiesen (1993) to ingest bacteria. However, *Prymnesium* also ingests larger prey such as dinoflagellates (e.g. *Oxyrrhis*) and green algae (Tillman, 1998). Under bloom conditions, cells of *Prymnesium* may also be present in the water column, but it is probably significant that in the only study where this has been examined carefully, Johnsen and Lein (1989) found cells in the water masses to be concentrated in the upper few metres of the water column. This suggests that the cells attach to the water surface like neustonic algae, using the haptonema.

We are not aware that any species of *Chrysochromulina* has been described as capable of attachment, and this feature may be a reliable distinguishing feature between *Prymnesium* and *Chrysochromulina*, which are otherwise very similar.

Ten species of *Prymnesium* have been described to date. Two of these, *P. minutum* N. Carter and *P. czosnowskii* Starmach, are poorly characterized and may be forms of other species. The type species, *P. saltans*, has not been characterized ultrastructurally, but it is likely to be identical to *P. parvum*.

Seven species have been studied ultrastructurally and these are discussed briefly below. Five of the seven species tested for toxicity proved toxic to *Artemia* or other organisms and until proved otherwise, a bloom of *Prymnesium* should always be considered toxic.

16.3.1.9 *Prymnesium annuliferum* Billard 1983

Scale structure: Cells covered with two types of scale (Figs. 16.8A–E). One type (Figs. 16.8A, B), which occurs in one or more layers next to the cell, is oval, plate-like, with an inflexed dorsal rim. It measures *c.* 0.49 µm × 0.4 µm. The ventral side (Fig. 16.8A) shows a pattern of radiating ridges arranged in four quadrants, with about 14–15 ridges per quadrant. The rim is *c*. 0.08 µm wide. Scales of the outer layer are oval, *c*. 0.53 µm × 0.39 µm (Figs. 16.8C, D). The ventral face (Fig. 16.8C) has ornamentation similar to that of the inner layers. The outer surface shows two to three concentric ridges (Figs. 16.8D, E), each *c*. 0.07 µm high, the outermost ridge forming the rim of the scale. The innermost ridge is variable in appearance, sometimes U-shaped or reduced to a single or two parallel ridges of variable length. In addition to these two types of scale, Fig. 17 of Billard (1983) shows two smaller plate scales, indicating the existence, perhaps, of a third type of scale. These scales are distinctly smaller than the plate scales mentioned in the diagnosis.

Distribution: France only.

Toxicology: No toxicity to *Artemia* was found (Fresnel *et al*., 2001).

16.3.1.10. *P. calathiferum* Chang et Ryan 1985

Scale structure: Cells covered with two types of scale (Figs. 16.9A–C). One or two layers of oval, flat scales (Figs. 16.9A, B) are located immediately outside the plasmalemma. The ventral surface of these scales (Fig. 16.9A) bears radiating ridges in quadrants, about 12 ridges per quadrant. The dorsal face (Fig. 16.9B) has a system of concentric fibrils and a wide inflexed rim (*c.* 1/3 of the scale radius). The scales

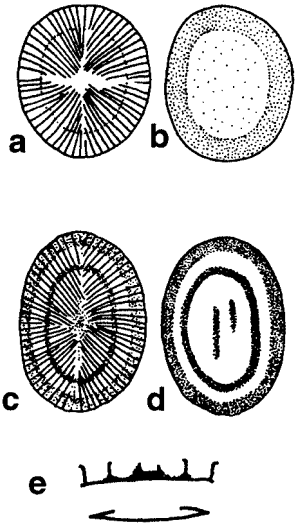

Figure 16.8
Scales of *Prymnesium annuliferum*. A, B, under-layer scales seen from the ventral and dorsal sides, respectively; C, D, scales from the outer layer seen from the ventral and dorsal sides, respectively; E, surface (top) and under-layer scales (below) in transverse section.
Source: originals, Moestrup and Thomsen.

measure *c.* 0.45–0.55 µm × 0.30–0.35 µm. The outer layer of scale (Fig. 16.9C) has an oval base-plate of similar size as the plate scales, but possesses an upright basket-like rim (*c.* 0.10–0.19 µm high), comprising both vertical and horizontal bars. A short pillar rises vertically from the centre of the scale and a network of fibrils extends from its top to the outer rim. The ventral face of the scale bears radiating ridges as in the plate scales.

Distribution: Currently known from New Zealand (Chang and Ryan, 1985), Viet Nam (Moestrup, unpublished), East Africa (Hansen, unpublished) and Martinique (Fresnel, Algobank, Université de Caen).

Toxicology: The toxin(s) are unknown, but *P. calathiferum* was tested toxic to fish (*Gambusia*) (Chang, 1985). It is considered responsible for fish and shellfish mortalities in New Zealand in 1983 (Chang and Ryan, 1985). In our studies, using *Artemia* on an isolate from New Zealand, no toxicity was noted (Moestrup, unpublished).

16.3.1.11 *P. faveolatum* Fresnel 2001

Scale structure: Cells covered with two types of scale (Fig. 16.14). Scales of the inner layers elliptical to oval, 0.38–0.41 µm × 0.28–0.30 µm, with radiating fibrils arranged in quadrants on both sides, about 15 fibrils per quadrant. On the dorsal side there is an inflexed rim and a central X-shaped figure along the intersection of the four quadrants. Single outer layer of elliptical scales, 0.30–0.36 µm × 0.25–0.27 µm. Similar to scales of the inner layers in having a pattern of radiating lines on both sides arranged in quadrants, about 15 per quadrant. However, there is an upright rim (0.07–0.08 µm high) and alveolate raised ornamentation on the dorsal side.

Distribution: Known from Malta, Greece, France and Spain (Fresnel *et al*., 2001).

Toxicology: Toxic to the brine shrimp *Artemia* (Fresnel *et al*., 2001).

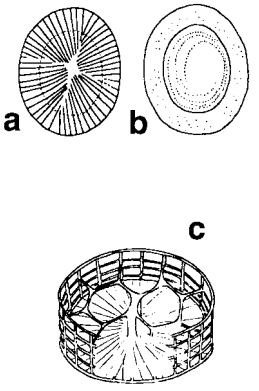

Figure 16.9
Scales of *Prymnesium calathiferum*. A, B, under-layer scales in ventral and dorsal views, respectively; C, scale from the outer layer.
Source: originals, Moestrup and Thomsen.

16.3.1.12 *P. parvum* N. Carter 1937

Scale structure: Cells covered with two types of scale (Figs. 16.10A–E). Scales of the inner layers are oval (Figs. 16.10A, B), 0.29–0.36 µm × 0.26–0.32 µm, with radiating ridges on the ventral side (Fig. 16.10A), and a pattern of concentric fibrils on the dorsal face (Fig. 16.10B), which has a wide (0.04–0.05 µm) inflexed rim. Scales of the outer layer very similar (Figs. 16.10C–E), 0.30–0.43 µm × 0.23–0.30 µm, but with a narrow rim only (Figs. 16.10C, D). The radiating ridges in both types of scale are arranged in quadrants, with about 11 ridges per quadrant.

Distribution: *P. parvum* is very widely distributed and known from temperate and subtropical regions of both hemispheres (Green *et al*., 1982).

Toxicology: *P. parvum* is a noted fish killer that is responsible for numerous fish mortalities, especially in low-salinity water (for a review see Moestrup, 1994; Johansson and Granéli, 1999; Guo *et al*., 1996, on cases in China). There has been extreme confusion regarding the chemical nature of the toxins and many different chemical compounds have been claimed to be responsible for the toxic effect: a proteolipid, a glycolipid, two galactolipids, a saponin, or two ethers. The most detailed recent work is that of Igarashi *et al*. (1999) who found two haemolytic and ichthyotoxic polyethers (glycosides), known as prymnesin-1 and -2. Both were complex structures based on a C_{90} carbon chain, and including five contiguous ether rings, three chlorine atoms and an amino group, in addition to 1-3 glycosidic radicals. The latter were identified as furanoses and a pyranose, and this part of the molecule differed between prymnesin-1 and prymnesin-1.

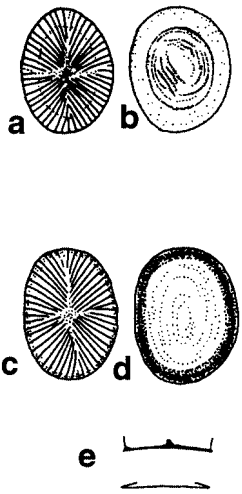

Figure 16.10
Scales of *Prymnesium parvum*. A, B, under-layer scales in ventral and dorsal views, respectively; C, D, scales from the outer layer in central and dorsal views, respectively; E, outer- (top) and inner-layer scales (below) in transverse section.
Source: originals, Moestrup and Thomsen.

16.3.1.13 *P. parvum f. patelliferum* (Green, Hibberd et Pienaar) A. Larsen 1999 = *P. patelliferum* Green, Hibberd et Pienaar 1982

Scale structure: Cells covered with two types of scale (Figs. 16.11A, B), both oval and measuring *c*. 0.36–0.37 µm × 0.25–0.27 µm. Innermost scales in up to three layers, each with a narrow inflexed rim and a central thickening on the dorsal face and a system of radiating ridges on both surfaces (Fig. 16.11A). The ridges are in quadrants with 11–13 ridges per quadrant. Scales of the outer layer similar but with an upright rim (Fig. 16.11B), *c*. 0.06 µm high.

Distribution: *P. patelliferum* (previously but incorrectly known as *P. patellifera*) is known from the west coast of the United States, France, the United Kingdom, Norway, Bulgaria, Australia (Green *et al*., 1982; Larsen and Moestrup, 1989; Larsen *et al*., 1993).

Toxicology: Toxic to the brine shrimp *Artemia salina* (Larsen *et al*., 1993; Larsen and Bryant, 1998) and to the dinoflagellate *Heterocapsa triquetra* (Arlstad, 1991). The organism responsible for a sudden mass mortality of fish in the Varna lakes in Bulgaria in 1959 may have been this species (Green *et al*., 1982).

Note: Larsen *et al*. (1993) and Larsen and Bryant (1998) found no marked differences in the growth rates of taxa identified as *P. parvum* and *P. patelliferum*. Larsen and Medlin (1997) examined cultures identified as *P. parvum* and *P. patelliferum* in detail and found no genetic difference in the ITS1 region of the two taxa, nor in the banding pattern of an intron in the calmodulin genes. It was therefore concluded that the two taxa belong to the same species. The oldest and valid species name for these is *P. parvum*. *P. patelliferum* was relegated to a form of *P. parvum*, *P. parvum* forma *patelliferum* (Green, Hibberd et Pienaar) A. Larsen 1999 (Larsen, 1999). In studies of ploidy, Larsen and Edvardsen (1998) found forma *patelliferum* to be haploid, while forma *parvum* was diploid or haploid, indicating that the two forms belong to the same haplo-diploid life cycle.

16.3.1.14 *P. nemamethecum* Pienaar and Birkhead 1994

Scale structure: Cells covered with three different types of scale (Figs. 16.12A–G). One type is confined to the haptonema surface (Fig. 16.12G), and covers the

Figure 16.11
Prymnesium patelliferum. A, under-layer scale in ventral view; B, scale from outer layer, dorsal view.
Source: originals, Moestrup and Thomsen.

haptonema as a sheath. These scales are elliptic, 0.6 µm × 0.3 µm, with radiating ridges visible on both surfaces, about 20 per quadrant. The dorsal surface has a narrow inflexed rim *c*. 0.03 µm wide. In transverse thin section they frequently appear more or less V-shaped. The cell body is covered with up to six imbricate layers of flat, oval scales, measuring *c*. 0.85 µm × 0.6 µm (Figs. 16.12D–F). The ventral face of each scale (Fig. 16.12E) bears a pattern of radiating ridges in quadrants, each quadrant with about 30 ridges. The dorsal surface (Fig. 16.12D) possesses a patternless inflexed rim, *c*. 0.04 µm wide, and radiating ridges in addition to a central corona-like structure of somewhat variable construction. The outermost layer of scales consists of elongate, oval scales, measuring *c*. 0.65 µm × 0.35 µm (Figs. 16.12A–C). Both sides show a pattern of radiating ridges in quadrants, about 20 per quadrant. A 0.11 µm high raised rim lines each scale (Figs. 16.12A, C), while the central part comprises a raised thickening (*c*. 0.14 µm high) that subtends branches alternately to each side (Fig. 16.12C). The 17–22 branches extend to the peripheral rim and measure *c*. 0.045 µm in height (Fig. 16.12C).

Distribution: Described from full salinity sewater near Cape Town, South Africa, and also found in Australia (Pienaar and Birkhead, 1994) and Denmark (Jensen, unpublished).

Toxicology: Neither the South African nor the Danish isolate was toxic to *Artemia* (Moestrup, unpublished).

16.3.1.15 *P. zebrinum* Billard 1983

Scale structure: Cells covered with two types of scale (Figs. 16.13A–E). Innermost scales flat, in one or two layers (Figs. 16.13D, 13E). Scales circular or slightly elongate, measuring *c*. 0.3 µm × 0.28 µm. The ventral face with a pattern of radiating ridges in quadrants, 10–11 ridges per quadrant (Fig. 16.13D). The dorsal face appears patternless (Fig. 16.13E), but has an inflexed rim *c*. 0.06–0.08 µm wide. Outer scales in a single layer, elliptic, *c*. 0.35 × 0.27 µm (Figs. 13A–C). The ventral face with radiating ridges in quadrants, 11–12 ridges per quadrant (Fig. 16.13C).

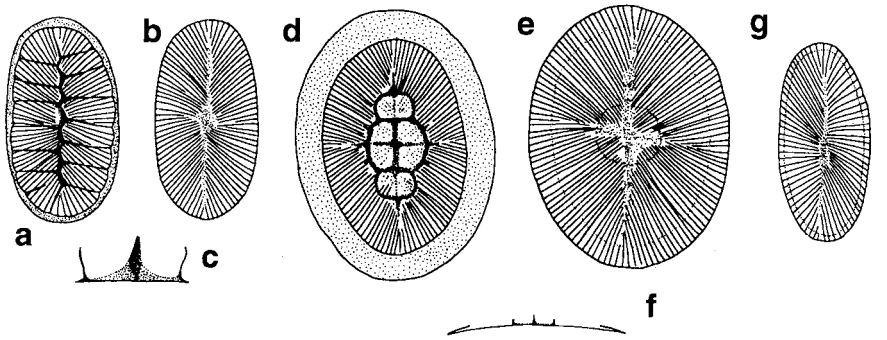

Figure 16.12
Prymnesium nemamethecum. Scales from the distal layer: A, dorsal view; B, ventral view; C, transverse section. Scales constituting the proximal layers: D, dorsal view; E, ventral view; F, transverse section. Scale from the haptonema sheath: G, dorsal view.
Source: originals, Moestrup and Thomsen.

The dorsal face with raised rim 0.05–0.06 µm high, and four to six parallel cross bars, each 0.025–0.03 µm high (Figs. 16.13A, B). The cross bars are arranged perpendicularly, or nearly so, to the long axis of the scale. The dorsal face otherwise appears patternless.

Distribution: Known from three locations in France only.

Toxicology: Toxic to *Artemia* (Fresnel *et al*., 2001).

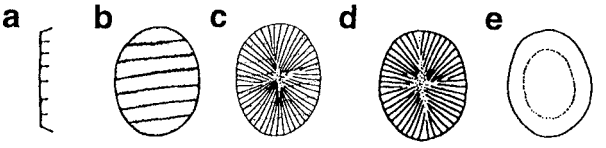

Figure 16.13
Prymnesium zebrinum. Scales from the outer layer: A, transverse section; B, dorsal view; C, ventral view. Scales from the innermost layers: D, ventral view; E, dorsal view.
Source: originals, Moestrup and Thomsen.

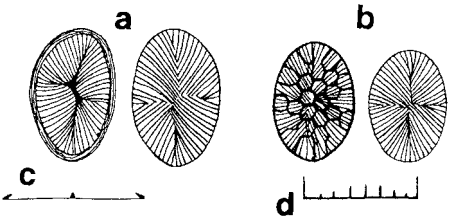

Figure 16.14
Prymnesium faveolatum. Scales from the inner layer: A, dorsal (left) and ventral view (right); C, transverse section. Scales from the distal layer: B, dorsal (left) and ventral view (right); D, transverse section. All magnifications × 40,000.
Source: Fresnel *et al.* (2001).

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17

Taxonomy of harmful diatoms

G. A. Fryxell and G. R. Hasle

17.1 Introduction

The diatoms are one of the largest algal groups known. Although traditionally 10,000–12,000 species are accepted, a much higher number has been suggested, e.g. Round and Crawford (1984, p. 169; 1989, p. 574) wrote that 'Experienced diatom systematists estimate a probable final number of approximately 50,000 species', and 'There are approximately 250 commonly recorded genera; the number of species described is in excess of 100,000'. Diatoms are found in all types of aquatic habitat, in the marine plankton at all latitudes, and through all seasons. Sournia *et al.* (1991) estimated between 1,365 and 1,783 diatom species (865–999 centric and 500–784 pennate species) to be from the marine planktonic habitat.

Although unicellular, many of the planktonic diatoms appear in various kinds of colony. Cells embedded in mucilage that is extruded from the diatoms are especially relevant when discussing harmful effects. Above all, the diatoms are characterized by their siliceous cell covering that contains most of the features used for identification to family, genus and species. Other distinctive features are cell shape, number and shape of chloroplasts, and structure of colony. These features are outlined below (Section 17.2.5).

This chapter deals with identification and taxonomy of those diatoms considered to be harmful. A few other related diatoms are included for comparison as an aid in supporting co-ordinated systems of integrated research efforts, which can result in early mitigation of harmful blooms (Millie *et al.*, 1999). Harm has been caused by blooms resulting in anoxic layers of water, production of oil or mucus, bitter taste in shellfish resulting in the loss of marketable products, detrimental mucilaginous aggregates, excessive epiphytic growth, consumption of nutrients needed in mariculture projects, or the production of toxin. In addition, recent work has indicated that the presence of some diatoms might influence the passage of other toxins into the food web (Windust *et al.*, 2000). When harmful effects are noted, a dominant species may be augmenting other effects. This is a new area yet to be explored both with dinoflagellate toxins and domoic acid (DA), the one toxin thus far traced to three genera of diatoms and an increasing number of species.

17.2 Methodology

17.2.1 Collection

Sampling with a tube or any kind of water bottle (Venrick, 1978) provides ample material for identification of diatoms occurring in bloom concentrations. In most

cases an additional sample collected by a fine-meshed net (Tangen, 1978) is required for preparing critical light microscopy and electron microscopy. Crude cultures, serial dilution cultures (Throndsen, 1978b) and unialgal cultures provide other means of obtaining material for careful identification.

17.2.2 Preservation

Although information on the protoplast is best obtained by examination of non-preserved material, identification of species will usually require further procedures, including preservation. The preservatives in most common use in marine phytoplankton investigations (Throndsen, 1978*a*) are formaldehyde and potassium iodide plus iodine (Lugol's solution). Slightly acid preservative is preferred to hinder the dissolution of the silica wall. If stored for a longer time (on a scale of months or years), the sample should not be too dense, and the sampling jar should be of glass with a non-metallic lid. Frequent openings of the sampling jar often seem to reduce the effectiveness of the preservation. If feasible, two aliquots of the original sample, one to be archived and left unopened, would therefore be advantageous.

17.2.3 Preparation

Examination of raw (uncleaned) material in water mounts gives sufficient information to identify some genera and most of their species, for example *Chaetoceros*, *Cerataulina*, *Guinardia*, *Leptocylindrus*, *Rhizosolenia* and *Skeletonema*. These genera are recognized by specific structures such as setae, elevations, and special shapes of the valve and the bands. Species identification of other diatoms, such as species of *Thalassiosira* and *Pseudo-nitzschia*, require examination of valve structure. As the refractive indices of silica and water are about the same, for the best contrast it is necessary to embed the diatom cell in a medium of a higher refractive index. The material to be embedded may just be rinsed to eliminate preservative and sea water or, in addition, may be cleaned to oxidize organic cell elements and to separate the various frustule components. A great number of methods for cleaning and preparing permanent diatom slides are in use (e.g. Hasle, 1978; Round *et al.*, 1990).

17.2.4 Microscopy

Most of the planktonic diatoms in question are weakly silicified. The use of phase or differential interference contrast (DIC) optics is recommended, especially for light microscopy of water mounts, but also for examination of cleaned material embedded in a medium of a high refractive index. Electron microscopy can ensure correct identification of some of the diatoms mentioned as harmful, especially the *Pseudo-nitzschia* species. Cleaned material for light microscopy (LM) can be used also for electron microscopy and mounted on a formvar-coated grid for transmission electron microscopy (TEM) and on a stub for scanning electron microscopy (SEM; Hasle, 1978; Round *et al.*, 1990). The transmission electron microscope is usually the best instrument for examination of the fine structure of the *Pseudo-nitzschia* valves, especially the striae.

17.2.5 Terminology and gross morphology

The shape of the external skeleton of a diatom, called a frustule, is usually compared with that of a box, with a larger half fitting over a separate, slightly smaller half. On

the half of the frustule with the larger diameter, the lid of the box is an element called a *valve* and the sides are made up of a series of bands that stay with the valve for the life of the cell. The smaller half of the frustule is made from similar parts, including another valve, with the exception that the sides of the box, or bands, are formed sequentially and slipped in place as the young cell matures and growth takes place before the next division cycle. All the bands together are called the girdle and the cell may be seen in girdle view or valve view (Fig. 17.1). The flattened part of the valve is called valve face and the rounded or steep peripheral part is called valve mantle. Valves as well as bands are usually not solid silica but have an open structure that lends strength without great weight. An individual opening, whether a pore or a chamber, is an areola. The areolae may be arranged in rows called striae. In centric diatoms the striae are oriented towards a point, and in pennate diatoms towards a line. (For further information see: Anonymous, 1975; Ross *et al.*, 1979; Round *et al.*, 1990, p. 4).

17.3 Diatom genera involved in harmful events

Most harmful events have been noted in coastal waters where nutrients are high and observations frequent. (Please check Appendix 17.1 for authorities for the diatom genera and species used in the text.)

17.3.1 *Coscinodiscus*

Coscinodiscus concinnus and *C. centralis* were found in May 1947 in the North Sea in concentrations discolouring the water and forming an oily film on the sea surface. This sticky oil adhered to birds' feathers and bird mortality was observed (Tåning, 1951; Grøntved, 1952).

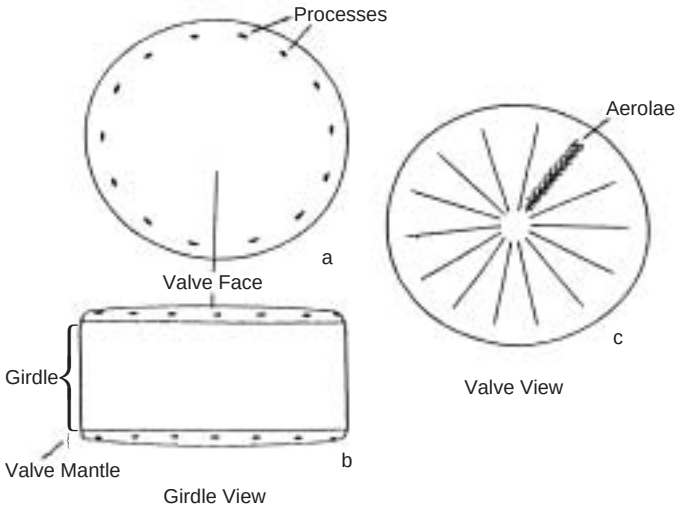


Figure 17.1
Centric diatom, schematic drawing, with terms. A and C, valve view; B, girdle view. Processes, or tubes through the valve, and areolae in patterns related to a point.

Coscinodiscus wailesii produces mucilage in cultures, and in the late 1970s the fishermen in the Plymouth area complained that their nets became clogged with a heavy jelly-like material. The mucilage from *Coscinodiscus wailesii*, an important constituent of the phytoplankton in these waters from 1977, and mucilage from the nets were shown to have basically the same chemical structure (Boalch, 1984). A dissolved oxygen deficit caused by the same species has been reported in the Seto Inland Sea of Japan (Manabe and Ishio, 1991), and in addition rapid consumption of nutrients from winter to spring that proved harmful to Nori (*Porphyra* C. Agardh) seaweed cultures (Nagai and Manabe, 1993) after rejuvenation of benthic resting cells (Nagai *et al.*, 1995).

17.3.2 *Thalassiosira*

Thalassiosira mala forms mucilage colonies and caused discoloured water, clogging the gills of cultured bivalves in Tokyo Bay in 1951 (Takano, 1956, 1965). The reported loss amounted to about 58 million yen (Takano, 1956, p. 65). In cell size and in shape of colonies, the diatom is similar to the prymnesiophyte, *Phaeocystis* Lagerheim, and the two may be confused.

17.3.3 *Cerataulina*

Cerataulina pelagica bloomed off the coast of north-eastern New Zealand in 1983, coincident with the death of benthic shellfish and bony fish, with deaths attributed to anoxia and clogging of the gills (Taylor *et al.*, 1985).

17.3.4 *Rhizosolenia*

Rhizosolenia chunii, tentatively identified, occurred in a bloom in Port Phillip Bay in south-eastern Australia from late August to mid-October 1987, coincident with the development of an unpleasant bitter taste of mussels, scallops and flat oysters, followed by high shellfish mortality three to eight months after the bloom had ceased (Parry *et al.*, 1989).

17.3.5 *Chaetoceros*

Chaetoceros spp. in large concentrations may clog the gills of farmed fish and also cause bleeding (Tangen, 1987, 1991, 1999; Horner *et al.*, 1990; Hallegraeff, 1993). This effect of *C. convolutus* had been observed on lingcod decades before, and the hypothesis was that the spiny *Chaetoceros* setae actually penetrated the gill tissue (Bell, 1961). Laboratory bioassays with Atlantic salmon and environmentally common (as well as greater) concentrations of *C. concavicornis* documented a physical action of the diatom on the fish gills. The gills started to produce mucus that induced hypoxia (deficiency of oxygen in the tissues of the body) and hypercapnia (excessive amount of carbon dioxide in the blood) (Rensel, 1993).

17.3.6 *Pseudo-nitzschia*

Pseudo-nitzschia species present in the plankton and the connection between the occurrence of amnesic shellfish poisoning (ASP) caused by the neurotoxin, DA, in shellfish and other marine animals have been studied intensively and an extensive literature on the subject exists (see: Bates, 1993; Bates, 2002, or as updated at that location.).

Pseudo-nitzschia multiseries (basionym, *Nitzschia pungens* f. *multiseries*) was blooming off Prince Edward Island, Canada, when the first recorded incident of ASP was documented in 1987 (Bates *et al.*, 1989). Three people died and others lost their short-term memory, apparently permanently. On 22 September 2000, harvesting molluscan shellfish from the Mill River area in western Prince Edward Island (PEI) was closed until 6 October because DA was found at a level of 33 µg per gram (net weight) of mussel tissue (Bates and Richard, 2000). It was the first closure due to DA in western PEI and the first anywhere in PEI since 1994, with a brief bloom of *Pseudo-nitzschia multiseries* held responsible (Bates and Richard, 2000). Domoic acid again caused extensive closures in mollusc harvesting in the Atlantic side of Canada in the spring of 2002 (S. Bates, personal communication, 'Backgrounder' web site, 2002), affecting bays of northern Prince Edward Island, New Brunswick to portions of the Baie des Chaleurs in Québec, and portions of Nova Scotia. This was the first time that domoic acid had caused closures in Canada in the spring. 'The culprit this time was *Pseudo-nitzschia seriata*…' Bates stated. An increase in *P. seriata* had been noted earlier, linking the presence of that species with domoic acid in some molluscs (Coutré *et al.*, 2001).

In September 1991 deaths of pelicans and cormorants in Monterey Bay, California, were caused by *Pseudo-nitzschia australis* (synonym *Nitzschia pseudoseriata* Hasle) in the food chain (Buck *et al.*, 1992; Work *et al.*, 1993). The toxin had been concentrated in the gut of filter feeders and Dungeness crabs, but was also found in the meat of anchovies and razor clams (Fritz *et al.*, 1992). *P. australis* was found in coastal waters of Galicia, north-western Spain, in 1996, and DA was detected in mussels (Miguez *et al.*, 1996). Later different strains of five *Pseudo-nitzschia* species were isolated and cultured by the same team; DA was detected in *P. australis* (Fraga *et al.*, 1998).

Recent observations of the phytoplankton-anchovy-mammal food chain of DA in Monterey Bay confirmed the source of the neurotoxin to be *P. australis*, killing 400 seals in May and June 1998, with other seals showing signs of neurological dysfunction (Lefebvre *et al.*, 1999; Scholin, 1999; Scholin *et al.*, 2000), the first proven occurrence of domoic acid transfer through the food chain to a marine mammal (Trainer *et al.*, 2000). Anchovies have a neurologic sensitivity to DA similar to that of mammals (Lefebvre, 2000).

DA outbreaks have also been reported from Korea and New Zealand. In April 1993, Lee and Baik (1997) found that blue mussels in Jinhae Bay, South Korea, concentrated DA during the occurrence of a spring bloom of *P. multiseries*. In New Zealand, scallops in Northland in 1993 had *P. australis* cells packed in digestive glands, and scallop harvesting has had closures every November–December (Rhodes *et al.*, 1998a). In November 1994 *P. pungens* and *P. turgidula* were associated with contamination of shellfish (Rhodes *et al.*, 1998a). Although *P. delicatissima*, *P. pseudodelicatissima* and *P. multiseries* have been implicated in DA found in shellfish, *P. australis* is the main cause of contamination in scallops in New Zealand (Rhodes *et al.*, 1998*b*).

Some harvesting closures of shellfish fisheries were implemented in 1998 around Scotland, but closures were far more extensive the year following. Bates (2000) states that this occurrence resulted in the world's largest area closed to shellfishing to date. Around 14,400 square miles of coastal waters on the west and north coasts of Scotland, as well as some islands, were closed to scallop fishing (from 7 July 1999, with some still closed in April 2000, and again for 22 fishing areas

along the west coast on 18 August 2000), because scallops from the area contained DA above quarantine levels (A. W. Berry, ASP notes in phycotoxin website; also Gallacher *et al.*, 2001). The King scallop, *Pecten maximus* (Linnaeus), had higher DA concentrations than the Queen scallop, *Chlamys* (*Aequipecten*) *opercularis* (Linnaeus), and whole animal samples tested higher than gonad samples. There have been severe economic consequences and disruption of a fishing industry worth more than £17 million annually. It was presumed to be caused by *Pseudo-nitzschia* spp., as affected by increase in winter water temperatures (4°C over last six years), resulting in reduced annual temperature range, and increasing wind speeds that enhance mixing and offshore circulation (Gallacher *et al.*, 2001). *Pseudo-nitzschia australis* was present and dominant in some Western Scotland ASP closure areas during 1999–2000, and *P. australis* cultures isolated from 1999 blooms did produce DA (Bolch, pers. comm.). There were seven other potentially toxic species present that may also have contributed to ASP closures in 1999 and 2000 (Dunstaffnage Marine Laboratory website, http://www.nerc.ac.uk /).

After sampling was begun in 1995, domoic acid in samples collected in 1998 from Cote d'Armor (English Channel) first showed traces of DA related to the presence of *P. pseudodelicatissima* (Amzil *et al.*, 2001). Since 1999, during blooms in months of April to August, DA assays in shellfish have been performed systematically in France along the Atlantic and Mediterranean coasts by the phytoplankton monitoring network when the number of *Pseudo-nitzschia* spp. rises above 100,000 cells per liter. In the year 2000 an increase in DA as noted, especially in shellfish from Western Brittany coast, where levels of 27–53 µg DA/g of meat were attributed to *P. pseudodelicatissima* and *P. multiseries* (Amzil *et al.*, 2001).

In the Bay of Fundy in 1988, a bloom of *Pseudo-nitzschia pseudodelicatissima* (synonym *Nitzschia delicatula* Hasle) was associated with DA in soft-shell clams, *Mya arenaria* Linnaeus, and blue mussels, *Mytilis edulis* Linnaeus. Areas of the Bay were closed to mussel collecting because of the concentration of DA, and cultures were shown to produce the toxin (Martin *et al.*, 1990; 1993). After 1988, no additional harmful events were reported from blooms of *P. pseudodelicatissima* until 1998, when domoic acid was detected in razor clams and shellfish harvesting was closed along the coast of Washington state (United States), with 70–100% *P. pseudodelicatissima* in the seawater sample (Adams *et al.*, 2000). Razor clams became toxic within 18 days of the appearance of the bloom, which was attributed to environmental conditions, but depuration was still not complete as of October 1999 (Adams *et al.*, 2000). However, apparently non-toxic blooms of *P. pseudodelicatissima* have been reported in Scandinavian coastal waters (Lundholm and Skov, 1993; Hasle *et al.*, 1996), Australia (Hallegraeff, 1994), California (Walz *et al.*, 1994), and the northern Gulf of Mexico (Parsons *et al.*, 1998). Not previously reported, in the Bioluminiscent Bay, Puerto Rico, on 30 December 1999, a bloom of *P. pseudodelicatissima* was seen that had disappeared by 3 January 2000 (Navarro, pers. comm.).

Beltrán *et al.* (1997) reported that 150 dead pelicans were found in a period of five days in January 1996, at Cabo San Lucas, Mexico, the tip of Baja California peninsula. Although all the stomachs of dead birds were empty, possibly indicating earlier vomiting, extracts from the digestive tracts indicated DA as a possible cause of death. In addition, microscopic examination of material from the pelicans and mackerel showed empty frustules similar to *Pseudo-nitzschia*. They also cited Morales Chávez *et al.* (1994) as describing the death of 110 pelicans at Ensenada, Baja California, Mexico, in 1992, although the cause of death was not found.

17.3.7 *Cylindrotheca*

Cylindrotheca closterium and *Pseudo-nitzschia* spp. dominated among the diatoms in the macroscopic mucilaginous aggregates in the Adriatic Sea in summer 1988 (Fanuko *et al.*, 1989; Stachowitsch *et al.*, 1990). This phenomenon, known locally as *mare sporco* (dirty sea), has been reported from as far back as 1723. In 1988 it appeared to such an extent that it detrimentally affected tourism and fisheries. The aggregates of various shapes, present at various depths, were considered originally to be produced by diatoms, predominantly of pelagic origin (Stachowitsch *et al.*, 1990). However, if any diatom could be considered predominant in 1988, it was, according to personal observation (GRH) of a net sample from Trieste, 16 August 1988, *Pseudo-nitzschia pseudodelicatissima* (see Section 17.3.6). Occurrences of 'mucilaginous aggregates' were again reported in mussels in a coastal area of the northern Adriatic Sea facing Emilia-Romagna in 1989 and 1991. The aggregates and mussels were analysed for ASP toxin with none detected (Viviani *et al.*, 1995).

17.3.8 *Tabularia*

Tabularia affinis was identified as the epiphytic diatom found in ribbon colonies adhering to the surface of cultured *Porphyra* seaweed in Japan from January to April 1995, apparently brought in on sea water (Nagai *et al.*, 1996). The resulting Nori products in the culture grounds of Hyogo Prefecture were discoloured and deteriorated, resulting in a financial loss of more than 2 million yen.

17.3.9 *Minutocellus*

Minutocellus pseudopolymorphus appeared in a dense bloom that was recorded for the first time at the beaches off Sylt, Schleswig-Holstein, Germany (IOC, 1999). This surf-zone bloom was restricted to the beach; it extended only about 20 m from the beach parallel to the coast over about 15 km. The water had a strong smell; visibility was reduced to less than 50 cm. Chemical analysis (high-performance liquid chromatography) of samples revealed no paralytic shellfish poison, diarrhetic shellfish poison, or ASP toxin (IOC, 1999). Hasle *et al.* (1983) assumed *M. pseudopolymorphus* to be benthic.

17.3.10 *Guinardia*

Guinardia delicatula (basionym: *Rhizosolenia delicatula*) was collected in April 2000 in a water sample that was almost like a monoculture. Fishermen's nets in Rosfjord, south-eastern Norway, were covered by a brownish mucilage, another example of clogging of nets (E. Dahl, pers. comm.).

17.4 Description of genera and species – centric diatoms – biddulphiales

The genera dealt with here have species that are considered harmful due to (a) mucilage production or anoxia (*Thalassiosira*, *Coscinodiscus*, *Chaetoceros*), (b) physical damage of the fish gills (*Chaetoceros*).

17.4.1 *Thalassiosira*

Type: *T. nordenskioeldii*

Morphology: *Thalassiosira*, a planktonic genus with more than 100 known species, has a worldwide marine distribution with a few species in brackish to freshwater. For terminology see Fig. 17.2. The drum-shaped cells generally occur in chains, the cells being connected by one or more chitinous threads extruded from strutted processes on the disc-shaped valves. These processes are somewhat more heavily silicified tubes through the valve and can often be seen in phase contrast in the light microscope as tubes or even as dots. *Strutted processes* (fultoportulae) are typical for the family Thalassiosiraceae. In contrast, a *labiate process* (rimoportula), also a tube through the valve and usually larger, is found in most centric diatoms (Fig. 17.3A). A third type of process is the *occluded process*, which has a long external tube that does not penetrate the valve wall and can be present in some species of Thalassiosiraceae.

About one-tenth of the known species of *Thalassiosira* are regularly found embedded as single cells or short chains in large gelatinous colonies (Figs. 17.3C, E).

Generic characters

- cells usually discoid to drum-shaped;
- many strutted processes with organic threads;
- one or a few labiate processes;
- areola cribrum ('sieve-plate') on inside wall.

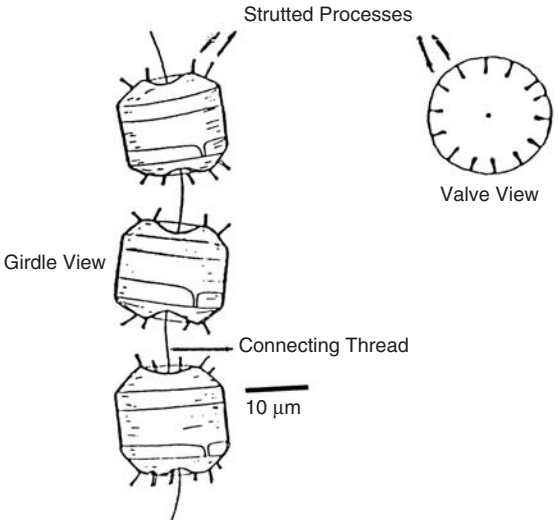


Figure 17.2
Thalassiosira with terms. Chain of three cells held together by thread(s). Valve view with one pattern of processes. Girdle view from Cupp (1943).

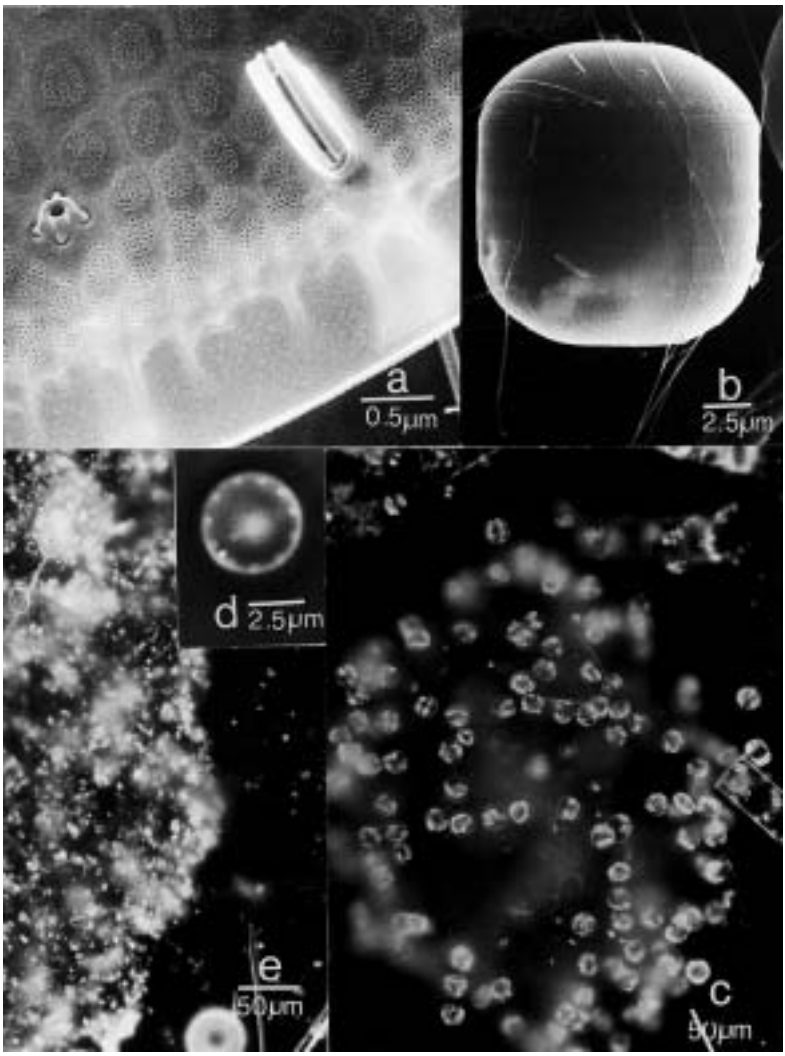


Figure 17.3
Thalassiosira
A, valve inside, with strutted process to the left, labiate process to the right, cribra, SEM.
B and C, *T. diporocyclus*
B, South Atlantic Ocean, *c.* 33° S., 18° E., SEM, whole cell with threads from strutted processes;
C, north-west Africa, phase contrast, colony.
D and E, *T. mala*, LM
D, Indian Ocean, phase contrast, valve with marginal processes, labiate process coarser and whiter than the strutted, central process to the left of more coarsely silicified central portion;
E, Trinidad, January 1968, phase contrast, part of colony and some free cells to the right.

Characters showing differences among species

- cell size;
- valve shape;
- areola pattern;
- number of areolae in 10 µm;
- process pattern;
- number of marginal processes in 10 µm.

Comments: The process pattern (Figs. 17.3B, D, as shown in Tables 17.1 to 17.3) is a useful tool for identification of the *Thalassiosira* species.

The shape of the gelatinous colonies is usually ill-defined and has been described as irregular gelatinous masses, cloud-like gelatinous masses, or just as cells embedded in mucilage. They have been found in high numbers even deep in the photic zone (>50 m) at a depth of sharply increasing density and nutrients (Gould and Wiesenburg, 1990). The chitinous threads (Fig. 17.3B), often with pennate diatoms on them (e.g. in Antarctic waters, Fryxell and Kendrick, 1988; fig. 2.5 in El-Sayed and Fryxell, 1993) have been seen as especially abundant in colonies of *T. partheneia*, *T. diporocyclus*, *T. fragilis*, and *T. minuscula* (synonym *T. monoporocyclus* Hasle) and one or more cells of *T. minuscula* have been seen inside some kind of capsules inside the colonies (Hasle, 1972*a*, figs. 46–48).

Under certain circumstances, more species than those mentioned here may form gelatinous colonies, as suggested in 1926 by Hustedt, who referred to findings of *T. nordenskioeldii* in this type of colony.

TABLE 17.1 Gelatinous colonies: *Thalassiosira* species with dome-shaped valves, radial lines of areolae arranged in sectors, with one central and at least one ring of strutted processes on the valve face in addition to a marginal ring

Species	Diameter (µm)	Areolae in 10 µm	Marginal ring of strutted processes, distance apart (µm)
T. diporocyclus	12–24	24–31	2.7–3.8, more crowded across from labiate process
T. tubifera	15–33	*c.* 20	3–4
T. subtilis	15–49	27–34	2.7–3.6
T. fragilis	19–30.5	40–50	3–5

Source: Hasle and Syvertsen, 1996, and sources within, plus unpublished observations.

Distribution: Northernmost and southernmost records
Somewhat larger species with dome-shaped valves
T. diporocyclus – c. 40° N. (Atlantic) – 40° S. (Pacific)
T. tubifera – c. 40° N. (Atlantic) – 05° S. (Pacific)
T. subtilis – c. 59–58° N. (Atlantic) – 47° S., Chile (Pacific)
T. fragilis – Gulf Stream warm core rings (Atlantic)

Species with flattened valves and external tubes of strutted processes
T. curviseriata – c. 59–58° N. (Atlantic) – c. 35° S., Australia (Pacific)
T. weissflogii – cosmopolitan (brackish water)
T. delicatula – c. 59–58° N. (Atlantic) – 47° S., Chile (Pacific)
T. gravida – cosmopolitan

Smaller species with rounded valves and no external tubes of strutted processes
T. mala – c. 59–58° N. – c. 35° S., Russian Federation, Africa, Brazil (Atlantic), Japan, Australia (Pacific)

TABLE 17.2 Gelatinous colonies: *Thalassiosira* species with one labiate process per valve and external extensions of strutted processes

Species	Diameter (µm)	Areolae in 10 µm	Marginal ring of strutted processes, distance apart (µm)	Areola patterns	Process patterns
T. curviseriata	5–14.5	20–30	3.8–4.9	Radial, but from offset centre	One to two eccentric strutted processes, two wings on marginal tubes
T. weissflogii	5–32	30–40	0.6–1.1, closely packed	Radial, areolae poorly developed sectors	Large labiate process, central ring of strutted processes
T. delicatula	9–30	22–26	2.0–2.5, three rings on mantle		Labiate process away from margin; occluded present with larger external tubes, one or more rings of strutted processes on valve face
T. gravida	17–62	*c.* 20	2–3?, several closely packed rings on mantle with longer external tubes	Radial, sectors	Large labiate process, central cluster of strutted processes with many on valve face, resembles a pincushion

Source: From Hasle and Syvertsen, 1996, and sources within, plus unpublished observations.

T. proschkinae – c. 59–58° N., Kristiansund, Norway, Caspian Sea, Azov Sea, estuaries of the Schelde and Elbe Rivers in Belgium/the Netherlands, English Channel, Germany, West Africa (Atlantic), Bering Sea, Japan (Pacific)

T. partheneia – c. 40° N. (Atlantic) – 40° S. (Pacific)

T. mediterranea – c. 59–58° N. (Atlantic) – 47° S., Chile (Pacific)

T. minuscula – c. 59–58° N. (Atlantic) – 47° S., Chile (Pacific)

Information on distribution is from Hasle (1972*a*, 1976, 1983), Rivera (1981), Takano (1981), Fryxell *et al.* (1984), Hallegraeff (1984), Mahood *et al.* (1986), Makarova (1988), Gayoso (1989), Lange *et al.* (1992), Licea (1994), Hernández-Becerril and Tapia Peña (1995), Harris *et al.* (1995), and Muylaert and Sabbe (1996).

17.4.2 *Coscinodiscus*

Lectotype: *C. argus*

TABLE 17.3 Gelatinous colonies: *Thalassiosira* species with a small, rounded appearance in girdle view, one ring of marginal strutted processes and one labiate process per valve, and without pronounced external tubes

Species	Diameter (µm)	Areolae in 10 µm	Marginal ring of strutted processes, distance apart (µm)	Areola patterns	Process patterns
T. mala	2–10	25–30	1–1.5	Centre coarsely silicified	Single strutted process on valve offset from centre
T. proschkinae	2–10.5	25–30	1.5–1.9	Radial	One areola between labiate and single central strutted process on valve face
T. partheneia	4.4–14	38–50	2.4–3.8	Radial, sectors	One central strutted process, longer internal tubes on strutted processes
T. mediterranea	6–20	*c.* 30	2–4	Areolae radial, usually poorly developed	Stellate central ring of strutted processes
T. minuscula	10–27	32–37	3.0–3.8	Sectors	Labiate process slightly away from margin; one central strutted process and one adjacent to labiate process on valve face

Source: Hasle and Syvertsen, 1996, and sources within, plus unpublished observations.

Morphology: The *Coscinodiscus* species generally occur as single cells, and they are larger in diameter and more coarsely structured than most *Thalassiosira* spp. They have no strutted processes or external tubes but at least one marginal ring of *labiate processes* (Table 17.4, Figs. 17.4B, C). The genus is marine and planktonic.

Generic characters

- cells discoid to cylindrical;
- no central process;
- many labiate processes;
- two larger marginal labiate processes;
- radial rows of areolae;
- areola cribrum ('sieve-plate') on valve wall outside.

Characters showing differences among species

- cell size;
- valve shape;
- height of mantle, measured as number of areolae between marginal ring of processes and mantle edge;
- number of areolae in 10 µm;
- areola pattern on valve face, e.g. fasciculation, spiralling rows;
- interstriae (unperforated radial areas bordering fascicles, or bundles of areola rows);
- structure of valve centre;
- process pattern.

TABLE 17.4 *Coscinodiscus* species forming harmful masses

Species	Diameter (µm)	Valve shape	Areolae in 10 µm	Areola patterns	Central area of valve	Labiate process ring(s) near margin	Labiate processes on valve face
C. wailesii	280–500	Flat valve face, mantle at right angle	5–6, smaller on valve face	Radial	Hyaline, irregular outline with extensions	Two, at juncture of valve face and mantle, 2–3 areolae from margin	Present
C. concinnus	110–500	Convex, slightly flattened in centre	7–9, smaller on mantle	Narrow fascicles, wavy appearance	Star–shaped, hyaline centre; rosette of larger areolae	One, 3–9 areolae from margin	None
C. centralis	100–300	Gently convex	4–6, smaller on mantle	Radial, fasciculated, or spiralling	Rosette of larger areolae	One, 3–4 areolae from margin	None

Source: from Hasle and Syversten, 1996, and sources within, plus unpublished observations.

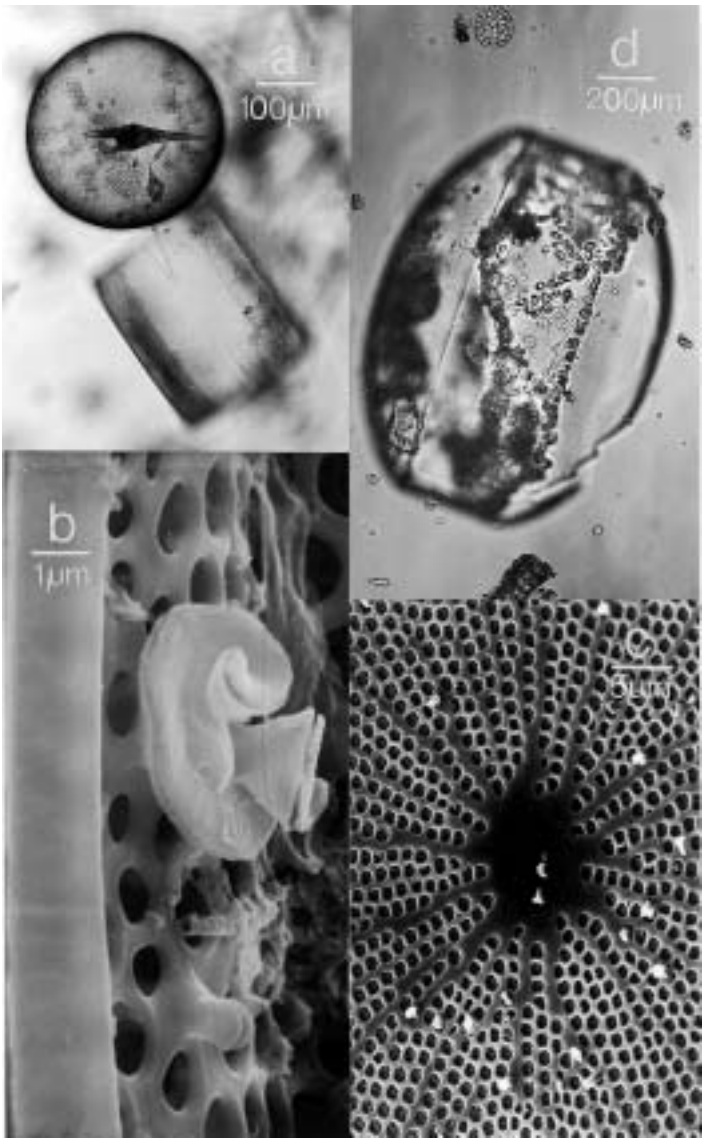


Figure 17.4
Coscinodiscus
A–C, *C. wailesii*
A, Skagerrak, NE Atlantic Ocean, phase contrast, whole cells in water mount in valve view (size compared with the dinoflagellate *Ceratium furca*), and girdle view;
B, Departure Bay, British Columbia, north-east Pacific Ocean, August 1928, SEM, larger and smaller labiate processes;
C, Culture from north-east Atlantic Ocean, harvested 18 August 1982, SEM, valve centre, labiate processes on valve face;
D, *C. concinnus*, Skagerrak, bright field, whole cell in water mount.

Comments: *Coscinodiscus wailesii* has a high, steep mantle with the two larger processes in the ring close to the valve edge (Figs. 17.4A, B). The species may thus be identified by fragments of the mantle, and with some experience, also by the c. 50 µm wide valvocopula having quincunx structure visible in the light microscope. *Coscinodiscus concinnus* (Fig. 17.4D) has more (but narrower) bands, and the valve has conspicuous interstriae running from the processes at a considerable distance from the valve edge towards the valve centre. *Coscinodiscus centralis* has the same number of bands as *C. wailesii*, but a shorter pervalvar axis, as each of the bands is narrower. The main distinctive characteristic of *C. centralis* is the central rosette of larger areolae.

Distribution:

C. wailesii – cosmopolitan, increase in abundance in North Atlantic waters since 1970s
C. concinnus – cosmopolitan
C. centralis – cosmopolitan

Information on distribution is from Boalch (1984); Rincé and Paulmier (1986); Hasle and Lange (1992, Table 3); Lange *et al.* (1992).

17.4.3 *Chaetoceros*

Lectotype: *C. tetrachaeta*

Morphology: *Chaetoceros* is one of the larger planktonic marine genera (approximately 175 species, Rines and Hargraves, 1988) with a worldwide distribution. The genus is recognized by its *setae* (or hollow outgrowths of valve projecting outside the valve margin, with structure different from that of the valve). For terminology see Figs. 17.5 and 17.6.

Generic characters

- chain formation by setae;
- two setae per valve;
- cells more or less rectangular in girdle view and elliptical to (rarely) circular in valve view.

Characters showing differences among species

- number of chloroplasts;
- terminal setae (setae of end valve of a chain) different or similar to the others in direction and structure;
- one seta of a cell longer than the three others;
- shape and size of the aperture (or ‘window’, the opening between adjacent cells);
- height of girdle compared with cell height (pervalvar axis);
- direction of chain (e.g. straight, curved, spiral).

The genus is divided into three subgenera: *Chaetoceros*, *Hyalochaete* and *Bacteriastroidea* (Hernández-Becerril, 1996), and the first two have been reported in harmful blooms. The species most commonly reported as harmful to fish belong to *Chaetoceros* (= *Phaeoceros* Gran).

17.4.4 *Chaetoceros* subgen. *Chaetoceros*

Morphology:

- numerous small chloroplasts throughout the whole cell, including the setae;
- large robust species;
- setae strong, thick, striated, armed with conspicuous spines;
- mostly oceanic species.

Comments: As the coarse, spiny setae of *Chaetoceros convolutus* and *C. concavicornis* are regarded as the injurious parts of the cell, the whole subgenus may be harmful to fish in net-pens. In waters of Washington State, Pacific coast of Canada, and South Chile, the harmful events caused by just these two species (and not by other species of the subgenus) may be due to the coincidence of geographical distribution of this subgenus and fish farming.

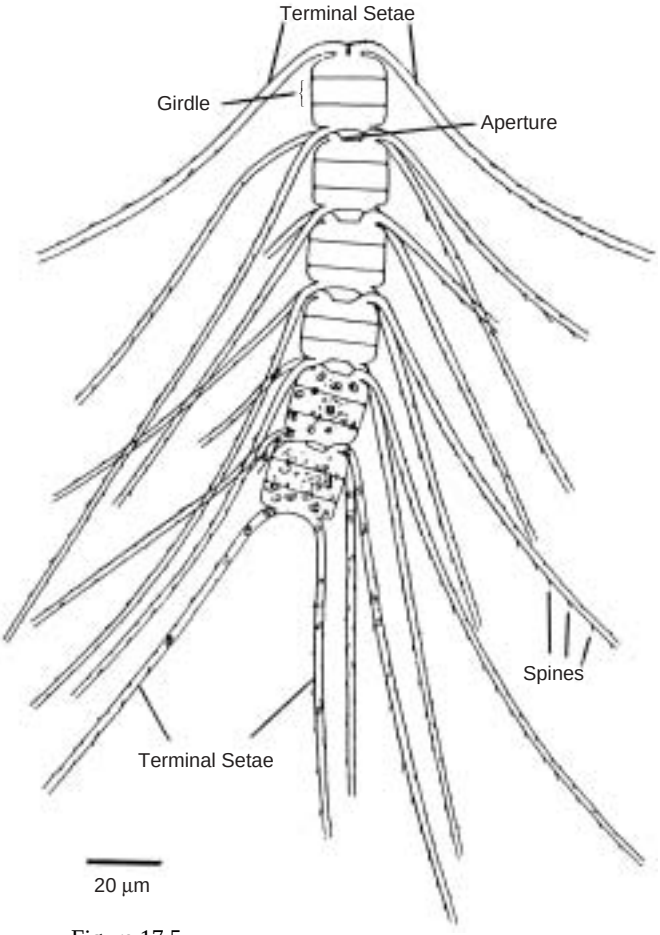


Figure 17.5
Chaetoceros convolutus, with terms.
Source: Hustedt (1930).

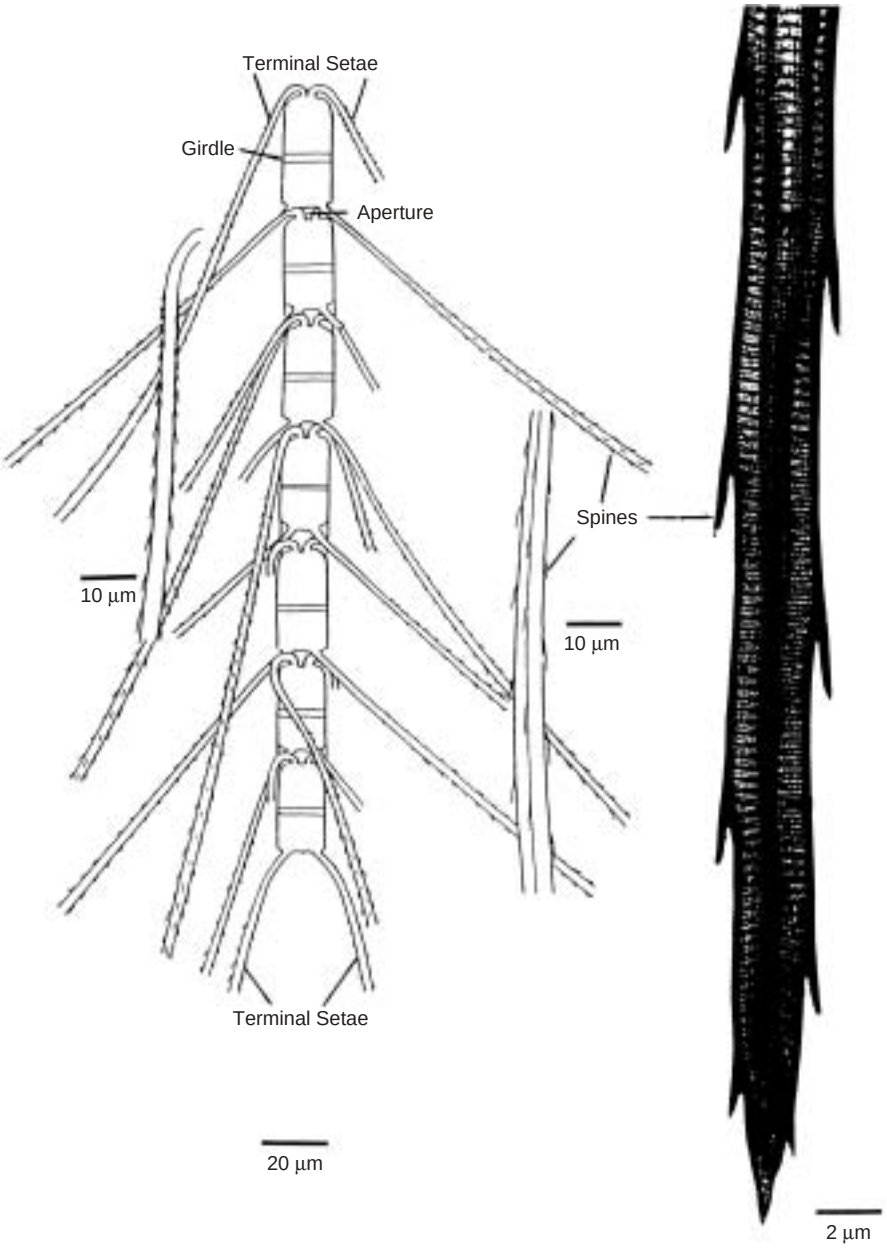


Figure 17.6
Chaetoceros concavicornis, with terms.
Seta, SEM. culture (D. Swift #189), from Gulf of Maine, north-west Atlantic Ocean.
Source: Hustedt, (1930).

Chaetoceros concavicornis and *C. convolutus* are differentiated by (a) height of the entire girdle and (b) thickness of the setae: the girdle of *C. concavicornis* being less than one-third of cell height and the girdle of *C. convolutus* being about the same height as each valve; and the setae of *C. concavicornis* increasing in width and in *C. convolutus* having the same width from the base and outwards (compare Figs. 17.5 and 17.6).

Distribution:

Cells solitary or in short chains
C. danicus – cosmopolitan
C. peruvianus – warm water to temperate
C. aequatorialis – warm-water region
C. rostratus – warm-water region
C. criophilus – southern cold-water region

Terminal setae differentiated from the others by length and direction
C. atlanticus – cosmopolitan
C. dichaeta – southern cold-water region

Terminal setae not distinctly differentiated from the others,
setae often diverging in all directions
C. borealis – cosmopolitan
C. concavicornis – cosmopolitan
C. convolutus – cosmopolitan
C. densus – cosmopolitan
C. eibenii – warm-water region to temperate
C. coarctatus – warm-water region
C. dadayi – warm-water region
C. tetrastichon – warm-water region
C. castracanei – southern cold-water region

The species listed here as cosmopolitan have probably their greatest abundances in temperate and/or cold waters.

17.4.5 *Chaetoceros* subgen. *Hyalochaete*

Morphology:

- one or a few plate-like chloroplasts;
- setae thin, often hair-like, without chloroplasts;
- spines mostly too small to be seen with LM.

Comments: This subgenus has the greatest number of species of the three now recognized (Hernández-Becerril, 1996). *Chaetoceros debilis*, the extract of which caused bradycardia (or slow heartbeat rate) on smolts of *Salmo salar* L. (Wildish *et al.*, 1991), and *C. socialis* belong to this subgenus. The harmful effect of *C. socialis* could just as well be caused by its presence in mucilage colonies as by the setae. *Chaetoceros debilis* is recognized by its curved and spirally twisted chains and almost rectangular apertures (as distinguished from *C. curvisetus* with elliptical to almost circular apertures). Intact spherical colonies of *C. socialis* may not be found in preserved material. The species is then recognized by three short setae of two adjacent valves and a fourth straight, elongated one. The latter entwines with the elongated setae of other chains to form a spherical colony.

Distribution: Greatest distribution in coastal waters
C. debilis – cosmopolitan, abundant in cooler waters
C. socialis – cosmopolitan

Information on distribution of *Chaetoceros* is summarized from Hustedt (1930), Hendey (1937, 1964), Cupp (1943), Rines and Hargraves (1988), Jensen and Moestrup (1998), and Hernández-Becerril and Granados (1998).

17.5 Description of pennate genera and species – Bacillariales

Toxin production (DA) has been found or imputed in a few genera (*Pseudo-nitzschia*, *Nitzschia*, *Amphora*).

17.5.1 *Pseudo-nitzschia*

Lectotype: *P. seriata*

Pseudo-nitzschia was established to include *Nitzschia seriata* Cleve, *N. fraudulenta* Cleve, and *Synedra sicula* Castracane (= *Nitzschia sicula*), was reduced to a section of *Nitzschia* by Hustedt (1958), but more recently has been recognized as a distinct genus (Hasle, 1994). Approximately 20 species share the most prominent ecological and morphological features of the type, *P. seriata*, and form a well-defined group demarcated from other genera of the family Bacillariaceae. The genus is marine and planktonic. For terminology see Fig. 17.7. Some species from the three genera in this section are considered harmful because of the production of the toxin DA (Tables 17.5 and 17.6).

Morphology:

Generic characters

- stepped chains formed by overlap of valve ends;
- cells strongly elongate, spindle-shaped or rectangular in girdle view, narrowly lanceolate to spindle-shaped or linear with rounded or pointed ends in valve view;
- valves shallow, flattened, weakly silicified;
- raphe, one or a system of two longitudinal slits through the valve wall, extremely eccentric, along one margin, not elevated above the general valve level;
- chloroplasts, two plates along the girdle.

Characters showing differences among species

- valve outline (margins curved or straight, can be one curved and the other straight in some species);
- shape of valve ends in valve and girdle views;
- size of central interspace or presence/absence of central nodule (or pseudonodulus in older literature);
- number of fibulae (or keel punctae in older literature) compared with number of interstriae (formerly transapical costae);
- cell length (apical axis);
- cell width (valve width, or transapical axis);
- cell height (or pervalvar axis).

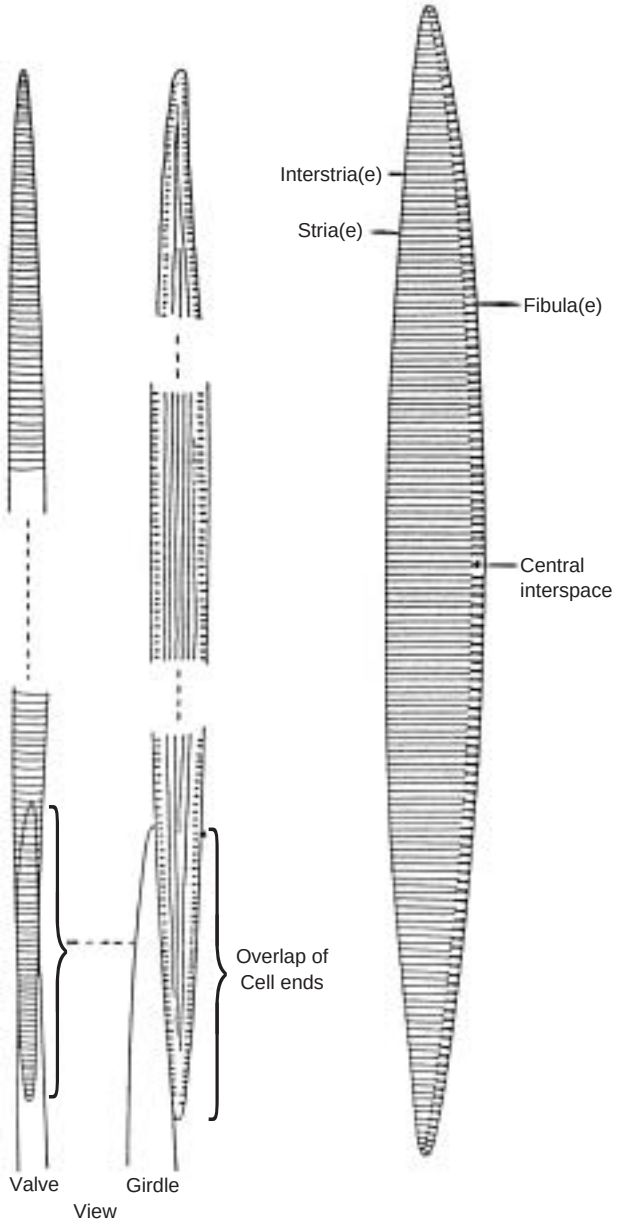


Figure 17.7
Pseudo-nitzschia, with terms.

TABLE 17.5 Morphology of *Pseudo-nitzschia* species that have been considered in domoic acid studies

Species	Apical axis	Transapical axis	Interstriae in 10 µm	Fibulae in 10 µm	Central larger interspace	Valve margins (plan view)
P. delicatissima	40–76	1.0–2.0	36–40	20–23	Present	Gently curved until some distance from poles
P. pseudodelicatissima	59–140	1.3–2.5	30–46	16–26	Present	Straight (linear) until some distance from poles
P. turgidula	30–80	2.5–3.5	23–28	10–13	Present	Truncated cell ends and short overlap in chains
P. pungens	74–174	2.4–5.3	9–16	9–16	Lacking	Linear or gently curved until pointed poles
P. multistriata	38–65	2.5–3.8	37–46	23–30	Lacking	Linear over much of valve, sigmoid near poles
P. multiseries	68–140	3.4–6.0	10–19	10–19	Lacking	Curved or almost straight; poles pointed
P. subfraudulenta	65–133	3.7–7.0	20–28	12–17	Present	Linear in mid-valve, pointed poles, symmetric
P. fraudulenta	50–119	4.5–10.0	18–24	12–24	Present	Fusiform, weakly silicified, interstriae faint
P. seriata f. *obtusa*	61–100	4.5–5.5	15–20	15–20	Lacking	One almost straight, one slightly curved; rounded poles
P. seriata f. *seriata*	91–160	5.5–8.0	14–20	14–20	Lacking	One curved, one almost straight
P. australis	75–144	6.5–8.0	12–18	12–18	Lacking	Curved to straight; poles slightly rostrate

Sources: Kaczmarska *et al.* (1986); Hasle and Syvertsen (1996); Skov *et al.* (1999); Rhodes *et al.* (2000); and references therein.

TABLE 17.6 *Pseudo-nitzschia* species from studies of domoic acid

Species	Positive domoic acid test?	Relative toxicity per cell	Toxic stage of growth	High abundance areas noted	Seasons of abundance	Selected references[a]
P. delicatissima	Yes	+	—	—	—	Smith *et al.* (1991)
P. pseudodelicatissima	Yes	+	—	North Atlantic, Gulf of Mexico Puerto Rico, California	Summer months	Martin *et al.* (1990); Hasle *et al.* (1996); Lundholm *et al.* (1997); Skov *et al.* (1999)
P. sp. cf. pseudodelicatissima	Yes	+	Exponential	—	—	Pan *et al.* (2001)
P. turgidula	Yes	+	—	South Pacific, New Zealand	—	Hasle (1965); Rhodes *et al.* (1996)
P. pungens	Yes	+	—	Temperate, coastal waters	Warmer months	Hasle *et al.* (1996); Rhodes *et al.* (1996)
P. multistriata	Yes	+	Stationary	Japan; New Zealand; Mediterranean Sea	Summer, winter	Takano (1993); Rhodes *et al.* (2000); Sarno and Dahlmann (2000)
P. multiseries	Yes	+++	Stationary	Canadian Maritimes, South Korea, Gulf of Mexico	Autumn, winter, spring	Bates *et al.* (1989); Fryxell *et al.* (1990); Villac *et al.* (1993*b*); Hasle (1995)
P. fraudulenta	Yes	+	—	New Zealand, Australia	—	Hasle (1965); Lundholm *et al.* (1994b); Rhodes *et al.* (1998*b*)
P. subfraudulenta	No	—	—	Gulf of Mexico, South Africa	Summer; winter in tropical waters	Hasle (1965); Parsons (pers. comm.)
P. seriata f. *seriata*	Yes	+++	Stationary	North Atlantic	Cold water	Hasle (1976); Lundholm *et al.* (1994*b*); Hasle *et al.* (1996)
P. australis	Yes	+++	Stationary? All?	Scotland, Galicia, West Coast United States, Chile	Autumn, spring	Buck *et al.* (1992); Villac *et al.* (1993*a*); Walz *et al.* (1994); Gallacher *et al.* (2001)

a. See also updates from Bates (2000, 2002).

Distinctive characters (Figs. 17.8–17.15):

P. delicatissima – cell ends rounded in valve view, cut-off in girdle view, short overlap (about one-ninth of cell length), with LM (acid-cleaned, mounted valves) fibulae visible, but not interstriae (Figs. 17.8D–F).

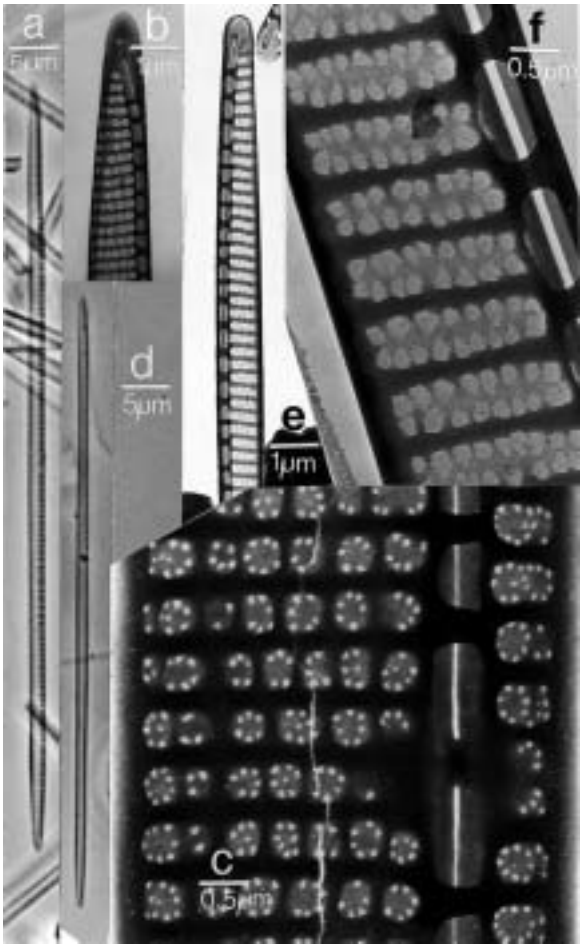
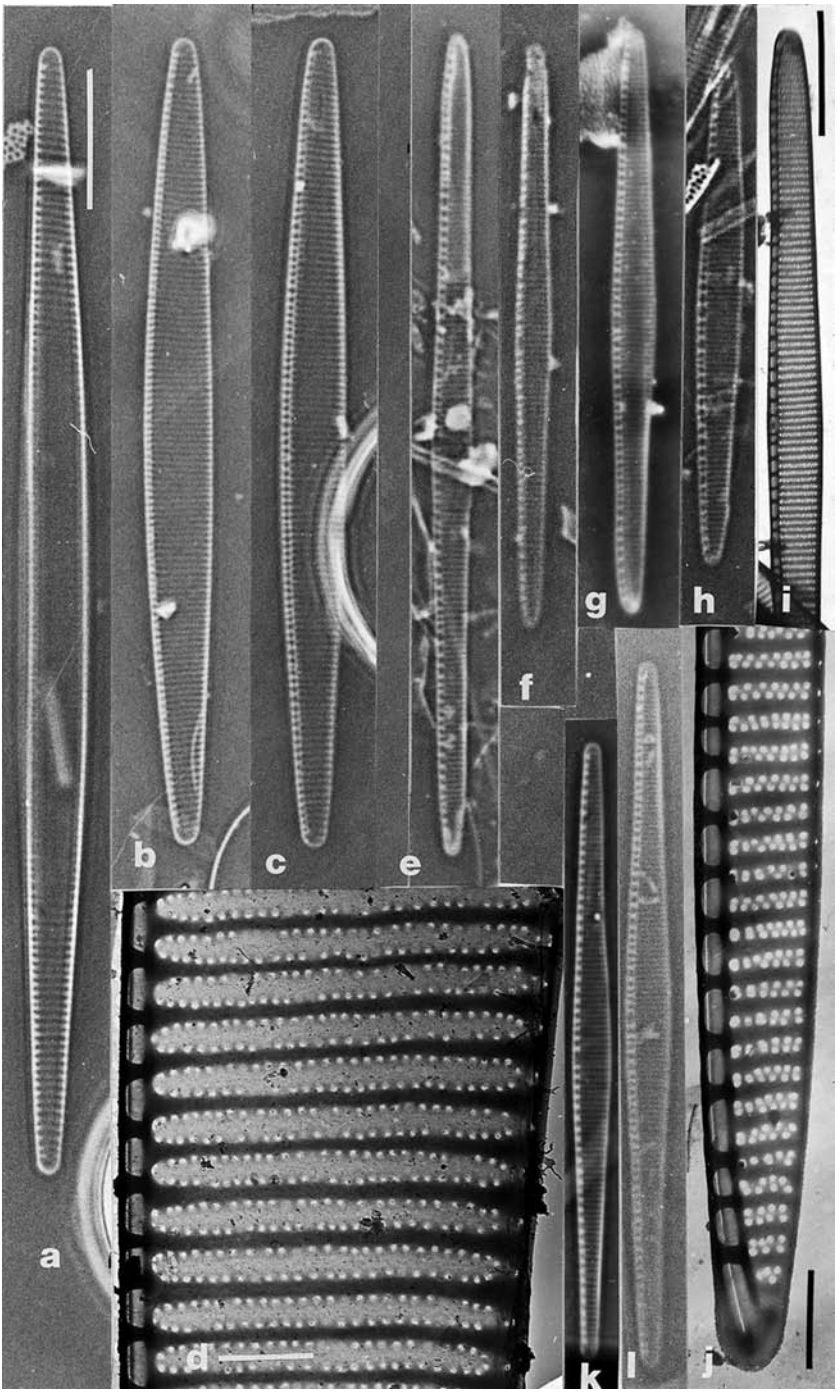


Figure 17.8
Pseudo-nitzschia delicatissima and *P. pseudodelicatissima*
A–C, *P. pseudodelicatissima* (can be toxic)
A, outer Oslofjord, Norway, September 1991, negative phase contrast, Naphrax mount, single valve, fibulae and central larger interspace visible;
B, Skagerrak, October 1991, TEM, pointed valve and striae with one row of areolae;
C, Skagerrak, October 1991, TEM, part of valve face, raphe with central larger interspace, central nodule, and valve mantle areolae structure.
D–F, *P. delicatissima* (can be toxic)
D, Skagerrak, May 1953, negative phase contrast, Naphrax mount, single valve, fibulae just discernible;
E, Skagerrak, May 1953, TEM, rounded cell end, striae with two rows of areolae;
F, Skagerrak, May 1953, TEM, part of valve face and raphe, areolae structure.

◄ Authors' note
Figure 17.8A-C shows *Pseudo-nitzschia calliantha* Lundholm, Moestrup et Hasle (erroneously identified as *Pseudo-nitzschia pseudodelicatissima*). See Lundholm *et al*. 2003 for recent information on the taxonomy and distribution of *Pseudo-nitzschia pseudodelicatissima*. Lundholm, N.; Moestrup. Ø.; Hasle, G. R.; Hoef-Emden, K. 2003. A study of the *Pseudo-nitzschia pseudodelicatissima/cuspidate* complex (Bacillariophyceae): what is *P. pseudodelicatissima*? *J. Phycol.* No. 39, pp. 797–813.

Figure 17.9 ➤
Pseudo-nitzschia seriata f. *obtusa* (A–C) and *P. turgidula* (E–J and possibly K, L), species with different structure and distributions. Raphes on left, scales = 10 µm for A-C, E-H, K, L; 1 µm for D, J; and 5 µm for I.
P. seriata f. *obtusa*
A, Kamchatka, 16 May 1955, phase contrast, illustrating a long valve somewhat similar to *P. seriata* f. *seriata,* but with less tapered poles;
B, coastal waters of northern Norway, 70°30′ N., 25°35′ E., 30 April 1962, phase contrast, holotype of taxon;
C, Kamchatka, phase contrast, valve with central irregularity but no central nodule;
D, Kabelvåg, northern Norway, *c.* 68° N., 3 March 1962, TEM, portion of valve with only two rows of poroids, in marked contrast to *P. seriata* f. *seriata.*
P. turgidula (rarely found to be slightly toxic)
E, South Pacific, 53°49′ S., 90° W., 13 December 1947, phase contrast, long valve showing slight swelling in mid-valve;
F, South Pacific, 55°50′ S., 90° W., 14 December 1947, phase contrast, shorter valve showing slight swelling in mid-valve and central nodule;
G, Indian Ocean, 49°26.6′ S., 132°18.2′ E., phase contrast, broader valve;
H, South Pacific, 53°49′ S., 90° W., 13 December 1947, phase contrast, shorter valve;
I, as above, TEM, detail showing valve structure and central nodule;
J, South Pacific, 52°50′ S., 90° W., 13 December 1947, TEM, pole of valve showing two closely packed rows of poroids.
Same species from the North Atlantic?
K, western Atlantic near Newfoundland, 49° N., 49°20′ W., 10 January 1968, phase contrast, valve very similar to *P. turgidula*;
L, Faerøe, 61°09′ N. 02° 19′ W., 9 September 1964, phase contrast, as above, with clear central nodule and swelling in mid-valve.


a
b
c
d
e
f
g
h
i
j
k
l

P. pseudodelicatissima – cell ends pointed in valve and girdle views, fibulae and larger central interspace distinct, interstriae occasionally visible in LM with acid-cleaned, mounted valves (Figs. 17.8A–C).

P. turgidula – short overlap of cells in chains (about one-sixth of length), margins in girdle view somewhat convex, tapering toward truncated ends; cell ends in valve view rounded, and smaller specimens lightly rhomboid to lanceolate, larger specimens linear, except for a middle expansion (Figs. 17.9E–J, also K, L?).

P. pungens – overlap of cell ends about one-third of cell length, coarsely silicified, interstriae visible in water mounts, fibulae visible as continuation of interstriae (acid-cleaned, mounted valves), perforation of striae mostly revealed in a medium of refractive index 1.6–1.7, phase contrast, objective oiled, × 100, n.a. 1.32 (Figs. 17.10A–C, 17.12C).

P. multiseries – interstriae visible in water mounts (uncleaned cells), fibulae more distinct than interstriae, perforation of striae not resolved with LM, irregularities often noted (Figs. 17.10D–F, 17.12C). *Pseudo-nitzschia pungens* and *P. multiseries* are now considered separate species because of morphological (Hasle, 1965, 1995) and genetic differences (Manhart *et al.*, 1995).

P. multistriata – overlap of cells in chains short; chains somewhat undulated, cells sigmoid in girdle view, poles slightly sigmoid in valve view, margins straight for the greater length of the valve, pointed poles, two morphotypes in original description (Figs. 17.11A–E).

P. seriata – overlap of cell ends about one-fourth to one-third of cell length, valve interstriae visible with LM (uncleaned cells in water mounts), raphe along either straight or curved margin, fibulae visible with LM as continuations of interstriae (on cleaned, mounted valves), difference in curvature of the two margins most expressed in middle valve (Figs. 17.12A, 17.13).

P. seriata f. *obtusa* – another cold-water species, is included here for comparison to show the blunt poles and different stria structure (Figs. 17.9A–C).

P. fraudulenta – overlap of cell ends in chains fairly short, fusiform in valve view, valve structure not discernible in water mounts, fibulae and central larger interspace distinct on cleaned valves in permanent mounts, interstriae sometimes barely visible in LM (Figs. 17.14A–C).

P. subfraudulenta – less frequently recorded than *P. fraudulenta*, mid-valves more linear, more than one interstria per fibula, in valve view the margins taper almost linearly near poles (Figs. 17.14D–G).

P. australis – valve middle part (about one-third of cell length) with straight to slightly curved margins, valve ends slightly rostrate with rounded poles (Figs. 17.12B, 17.15).

Comments: Increasing numbers of *Pseudo-nitzschia* species are being found to produce DA under certain conditions (Skov *et al.*, 1999). *Pseudo-nitzschia multiseries* and *P. australis* have been documented by observations as the sources of DA in the field as well as by studies of cultures (Garrison *et al.*, 1992; Villac *et al.*, 1993a, 1993b; Kotaki *et al.*, 1999; Parsons *et al.*, 1999; Lapworth *et al.*, 2000; Bolch, pers. comm.) and are generally considered the most likely to cause ASP.

Other findings conflict and suggest that different genetic strains or controlling ecological conditions are involved. Cultures of *Pseudo-nitzschia pseudodelicatissima* from the south-western Bay of Fundy produced DA, although at very low

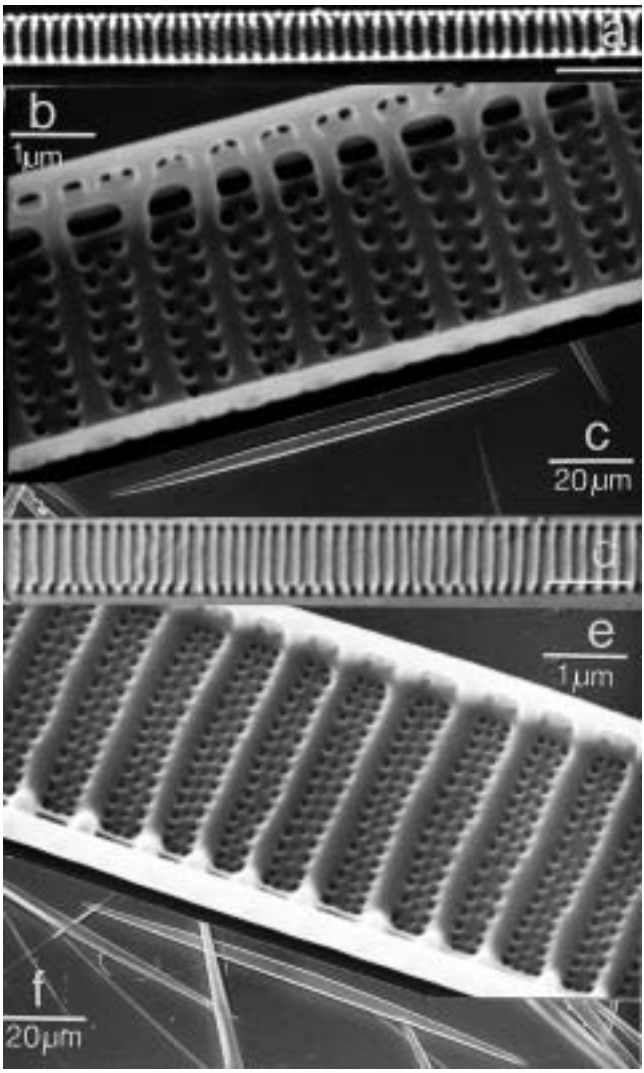


Figure 17.10
Two similar species of *Pseudo-nitzschia*
A–C, *P. pungens* (rarely found to be slightly toxic).
Scale = 5 µm
A, Skagerrak, North Atlantic, 12 September 1990, DIC, areolate striae visible;
B, Gulf of Mexico culture F242, SEM, two rows of striae between interstriae;
C, orientation view of valve in B, SEM.
D–F, *P. multiseries* (can be toxic)
D, Skagerrak, North Atlantic, 7 December 1989, LM, areolate striae not visible. Scale = 5 µm;
E, Gulf of Mexico culture MD-2, SEM, three (or more) rows of areolae between interstriae;
F, orientation view of valve detail in E.

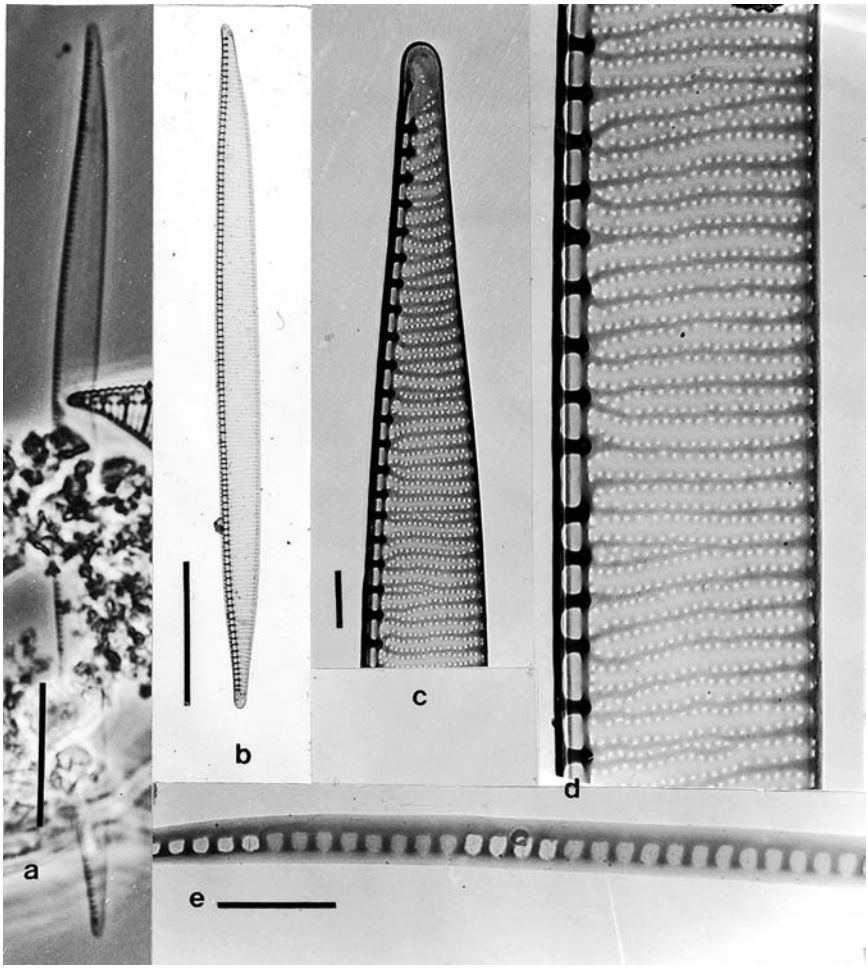


Figure 17.11
Psuedo-nitzschia multistriata (can be toxic). Raphes on left of valve views (A–D). TEM views (B-E), courtesy of N. Lundholm
A, *P. multistriata*? Gulf of Thailand, 27 September 1992, LM, negative phase contrast, only fibulae visible, may be broken. Note sigmoid poles. Scale = 10 µm;
B, culture from Port Shelter, Hong Kong, isolated by Yang Zhenbo and reisolated by N. Lundholm, smaller valve showing straight margins over much of the valve and slightly sigmoid poles. Scale = 10 µm;
C, culture as above, pole and fine structure of valve, with each fibula usually attached to two interstriae, often giving alternate striae similar outlines. Scale = 1 µm;
D, culture as above, fine structure of valve, with two rows of pores (11 in 1 µm) between two interstriae, similar to one original figure (fig. E, Takano, 1993). Scale as for E;
E, girdle band from same culture, TEM, with 46 areolae in 10 µm. Scale = 1 µm.

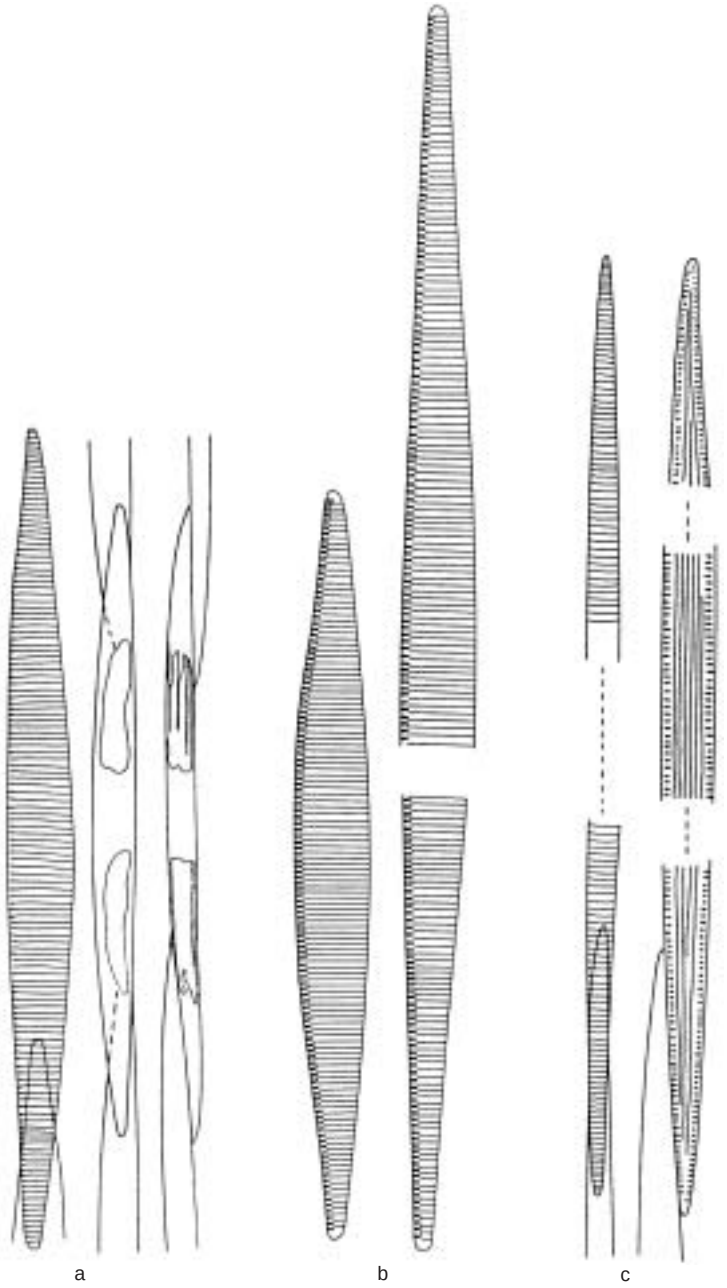


Figure 17.12
Three species of *Pseudo-nitzschia* that can be toxic: A, *Pseudo-nitzschia seriata*; B, *P. australis*; C, *P. multiseries* or *P. pungens* (as they would be indistinguishable at this level of detail).
Source: Hasle (1972*b*).

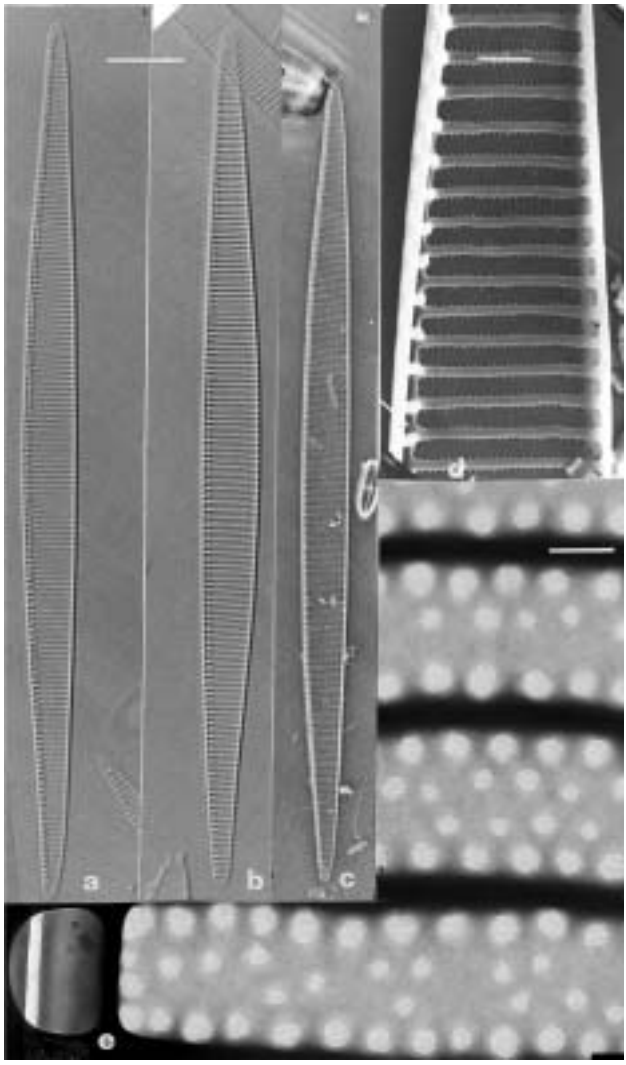


Figure 17.13
Pseudo-nitzschia seriata f. *seriata*, often confused with other species, but with limited latitudinal distribution. Raphes on left, scales = 10 µm for A–C; 1 µm for D; and 0.2 µm for E. No central nodule.
A, B, Inner Oslofjord, Norway, *c.* 59°40′ N., 60°35′ E., DIC
A, raphe on more curved margin;
B, raphe on straighter margin;
C, English Channel, 50°02′ N., 04°22′ W., phase contrast, specimen with fine structure, from one of the southernmost records;
D, Risør, southern Norway, *c.* 58°45′ N., 09°10′ E., SEM, three to four rows of poroids in contrast to the two rows seen in *P. australis*;
E, Saltfjord, northern Norway, 67°19′ N., 14°22′ E., TEM, showing structure of vela covering poroids.

concentrations (Martin *et al.*, 1990). Examination of an isolate of *P. pseudodelicatissima* from Danish waters grown at selected combinations of temperature and salinity detected DA on a few occasions (Lundholm *et al.*, 1997). Pan *et al.* (2001) found DA produced in exponential (instead of stationary) growth phase by cultures of *P.* sp. cf. *pseudodelicatissima* from the northern Gulf of Mexico. In contrast to those findings, the species was not found to produce DA in Danish (Lundholm and Skov, 1993), Australian (Hallegraeff, 1994), and Californian (Walz *et al.*, 1994) waters.

Smith *et al.* (1991) found that *P. delicatissima* (synonym *Nitzschia actydrophila* Hasle) appeared to produce DA under culture conditions, and Lundholm *et al.* (1994*a*, 1994*b*) found the same for *P. seriata*. Trainer *et al.* (1998) detected domoic acid in *P. pungens*. In New Zealand, where *Pseudo-nitzschia* blooms are common from spring to autumn (Rhodes *et al.*, 1998b), DA was detected in *P. pungens* and *P. turgidula* from field and culture studies (Rhodes *et al.*, 1996), although from later work there may have been some uncertainty about the identification of the latter. Rhodes (1998) found the highest level of DA per cell in *P. australis*; lower amounts in *P. pungens* and *P. multiseries*; small amounts in *P. fraudulenta*, *P. turgidula/delicatissima*; and *P. pseudodelicatissima*, and none in *P. heimii* and none also in a then-unidentified sigmoid *Pseudo-nitzschia* species.

The latter was identified as *P. multistriata* (Rhodes *et al.*, 2000), showing material resembling fig. E in the original description by Takano (1993). Sarno and Dahlmann (2000), showing material resembling fig. D in Takano (1993), report the production of DA by a small, sigmoid diatom also identified as *P. multistriata* from the Gulf of Naples (Mediterranean Sea). The size of the poroids (a feature considered conservative by Villac, 1996), from New Zealand and the Mediterranean Sea differ from those stated in the original description, but not from the original figures (Takano, 1993). Considering the type figures, there can be little doubt about the identification of the toxin-producing Mediterranean species (Sarno and Dahlmann, 2000), as well as an isolate that did not produce DA from New Zealand (Rhodes *et al.*, 2000). Future nomenclatural and laboratory work may clarify the nature of the differences in morphology and toxin-producing capabilities.

Distribution: Northernmost and southernmost records

P. australis – c. 56º27′ N., west Scotland – c. 40º S., Argentina (Atlantic), 54°05′ N., Gulf of Alaska – 56º19′ S., Chile (Pacific)

P. delicatissima – 79º N., Kings Bay, Svalbard – 32º S., State of Rio de Janeiro, Brazil (Atlantic); c. 37º N., Monterey Bay, California – c. 40° S., Tasmania (Pacific)

P. fraudulenta – 67º19′ N., Saltfjord, Norway – 38–40º S., Argentinean waters (Atlantic), c. 48º30′ N., Friday Harbor, Puget Sound, Washington – 53º50′ S., Chile (Pacific)

P. multiseries – 62º45′ N., Norwegian Sea – 38º30′ S., Quequen, Argentina (Atlantic), 60–50º N., west coast of Bering Sea, Kamchatka, Sakhalin Island – c. 35º S., Northland, New Zealand (Pacific)

P. multistriata – Gulf of Naples (Atlantic) – Fukuoka Bay and Tokyo Bay, Japan – northern waters of South Island, New Zealand (Pacific)

P. pseudodelicatissima – 67º N., Denmark Strait – c. 38º S., Argentinean waters (Atlantic); 69º38′ N., Herschel Island, Beaufort Sea – 53º13′ S., Chile (Pacific)

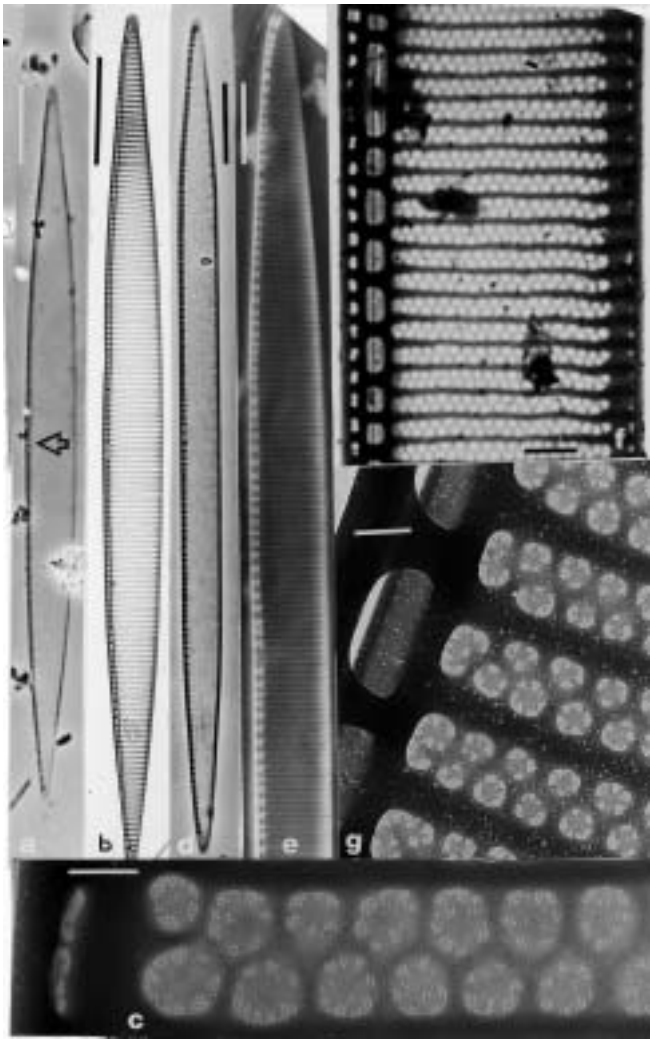
P. pungens – 63º25′ N., Trondheimsfjord, Norway – 36–48º S., Argentinean continental shelf waters (Atlantic); 60–50º N., west coast of Bering Sea, Kamchatcha, Sakhalin Island – region: – 49º52′ S., Chile (Pacific)

P. seriata – 77º44′ N., off Svalbard – c. 45º N., edge of continental shelf, east coast of the United States, between New York and Maryland (Atlantic)

P. seriata f. *obtusa* – c. 79° N., Kings Bay, Svalbard – c. 51° N., Gulf of St Lawrence (Atlantic); 49°47′ N., off Kamchatcha and Kuril Island (Pacific)

P. subfraudulenta – c. 43º19′ N., off Marseilles, Mediterranean Sea – 34º21′ S., south of Cape Town, South Africa (Atlantic); 27º10′ N., Gulf of California — c. 38º S., Tasmania (Pacific)

P. turgidula – c. 61º N., off Shetland Island? (Atlantic); c. 30–34º S., Australian waters – 53–62º S., South Pacific



The diatoms not found associated with DA and ASP but included here for comparison are *P. seriata* f. *obtusa* and *P. subfraudulenta*. Information on distribution is from Hasle (1965, 1972b, 2002); Takano and Kuroki (1977); Lange (1985); Rivera (1985); Kaczmarska *et al.* (1986); Takano (1993); Villac *et al.* (1993a); Hallegraeff (1994); Hasle and Heimdal (1998); Hasle *et al.* (1996); Hernández-Becerril (1998); Orlova *et al.* (1998); Rhodes *et al.* (1998b); Bérard-Therriault *et al.* (1999); Ferrario *et al.* (1999); Lapworth *et al.* (2000); Licea *et al.* (2000); Odebrecht *et al.* (2000); Orlova *et al.* (1998, 2000); Rhodes *et al.* (2000); Villac and Tenenbaum (2000); Sarno and Dahlmann (2000); Hasle (unpublished data); Hargraves (pers. comm.); Lapworth (pers. comm.); Lundholm (pers. comm.).

17.5.2 *Nitzschia*

Type: *Nitzschia sigmoidea*
Nitzschia navis-varingica

A pennate diatom in yet another genus has recently been isolated from a shrimp pond in Viet Nam and found to produce DA at levels reported for *Pseudo-nitzschia multiseries* (Kotaki *et al.*, 2000) and described as a species of *Nitzschia* (Lundholm and Moestrup, 2000). Cells are generally single but occasionally occur as doublets or triplets, adhering to each other by their valves, especially during rapid reproduction. Occasionally cells adhere to each other, girdle to girdle, but this was not interpreted as colony formation. The cell is 45–55 µm long, 9–11 µm wide, and rectangular in girdle view with a slight indentation in the centre. The pervalvar axis is wider than the transapical axis. Fibulae number 10–12 in 10 µm, and uniseriate

◀ Figure 17.14
Pseudo-nitzschia fraudulenta (A–C) compared with *P. subfraudulenta* (D–G). Raphes on left, scales = 10 µm for A, B, D; 5 µm for E; 0.2 µm for C, G; and 0.5 µm for F
P. fraudulenta (rarely considered slightly toxic)
A, north-west Africa, 17°45′ N., 16°30′ W., negative phase contrast, arrow pointing to central nodule;
B, Trondheimsfjord, Norway, culture, 23 October 1992, TEM, with almost symmetrical margins and gradual tapering from valvar midline to poles;
C, Heligoland, North Sea, 30 June 1992, TEM, with stellate pattern of electron-dense material surrounded by vela covering poroids and detail along raphe aligned with striae.
P. subfraudulenta
D, off Cape Town, South Africa, 34°31.8′ S. 18°24.8′ E.,
12 September 1961, negative phase contrast, valve with parallel sides over greater length and clear central nodule;
E, Panama City, Gulf of Mexico, 30°65′ N., 85°30′ W.,
22 December 1964, phase contrast, showing valve structure with clear central nodule and tapering near pole;
F, off Cape Town, as above, TEM, showing detail of valve structure with raphe slit under fibulae and central nodule near top left;
G, off Portugal, 41°50′ N., 08°54′ W., 23 November 1985, TEM, showing vela with quite similar pattern to *P. fraudulenta*, but no similar alignment of detail along raphe.

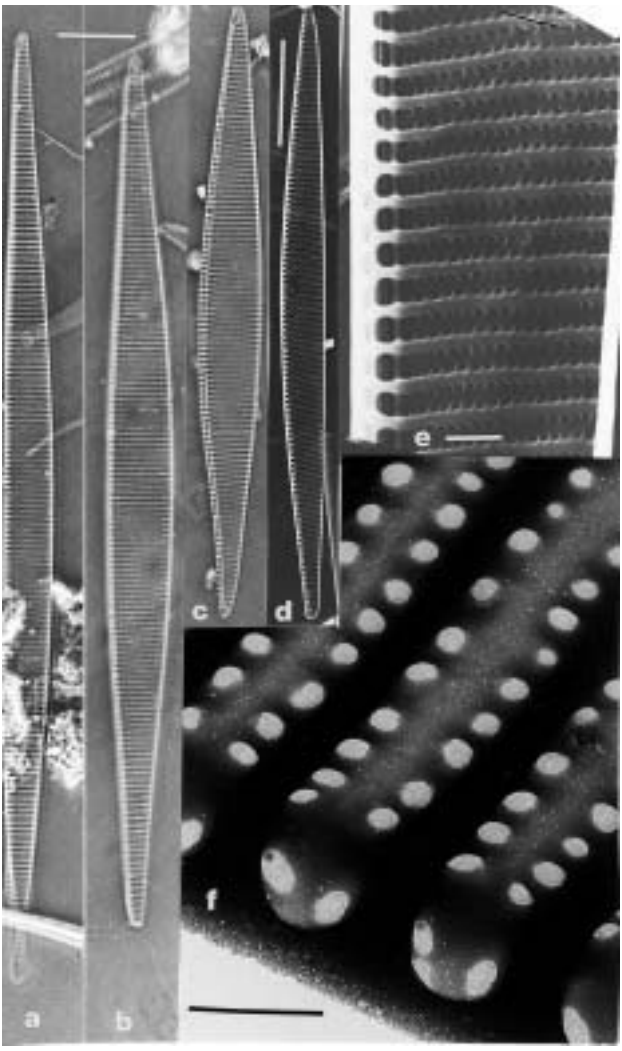


Figure 17.15
Pseudo-nitzschia australis from widely differing locations. Raphes on left. Scales = 10 µm for A-D; 1 µm for E; and 0.5 µm for F. No central nodule
A, Friday Harbor, Washington State, *c.* 48°36′ N., 123°06′ W., 1928, phase contrast, showing a long, slender specimen similar to *P. seriata*;
B, Ria de Pontevedra, north-west Spain, 24 April 1995, phase contrast, illustrating another long specimen;
C, Elbow Harbour, Cape Town, South Africa, 33°54′ S., 18°29′ E., 8 January 1993, phase contrast, shorter specimen;
D, Monterey Bay, California, culture MB-9, 12 February 1992, SEM, with asymmetrical shape;
E, same specimen as D, SEM, showing typical two rows of poroids;
F, Monterey Bay culture MB-9, TEM, showing vela covering poroid openings.

striae are 26–30 in 10 µm. The valve has a weak longitudinal fold and a moderately eccentric raphe. Although the species sinks in cultures, whether the species is planktonic or benthic in the field is uncertain. Shrimp ponds are very shallow, and benthic species are often found in the water column.

17.5.3 *Amphora*

Type: *A. ovalis*

Amphora coffeaeformis has also been reported as producing DA (Clone BPT 11: Shimizu *et al.*, 1989). Unfortunately, that clone is no longer available for observation, and other strains identified as the same species that have been tested have not produced DA (Bates *et al.*, 1989). Sala *et al.* (1998) indicated that several taxa have similar valve outline and dimensions and could be confused. The identity of the toxigenic species is uncertain, as Sala *et al.* conclude. It is apparent that taxonomic work is needed with this common group, as well as further testing of production of DA.

17.6 Concluding comments

Examples have been cited of bird mortalities, dissolved oxygen deficits, clogging of gills and of nets, bitter taste of shellfish, laceration of gills, mucilaginous aggregations, deterioration of commercial macroalgal products, strong smell and toxin production. However, any diatom frequently occurring in bloom proportions may be regarded as potentially harmful. Reared fish lose appetite and the power of resistance against infections when exposed to the regularly appearing abundance of *Skeletonema costatum* along the Norwegian coast (Tangen, 1999). A similar effect has been observed occasionally in South Chile with high concentrations of *Chaetoceros socialis*, which forms globular gelatinous masses. Fish kills were associated with high concentrations of *Leptocylindrus minimus* off central Chile, although the cause of the apparent noxious effect of the diatom could not be established (Clement and Lembeye, 1993).

It has been proved that DA is harmful to humans, mammals, birds and anchovies. The genus *Pseudo-nitzschia* has at least nine species that have been found to produce the toxin under some conditions. Domoic acid has been found when no *Pseudo-nitzschia* bloom has been known to occur (razor clams, *Siliqua patula* Dixon: Horner *et al.*, 1993; Pauley *et al.*, 1993; Bates, 1997), although samples might not have been taken in a timely manner, or depuration might be slow (Adams *et al.*, 2000). Species of both *Amphora* and *Nitzschia* have now been shown to produce the neurotoxin, and so awareness of the potential harmful effects of other pennate diatoms must be increased. *Pseudo-nitzschia* and *Nitzschia* belong to Bacillariales Hendey, and *Amphora* belongs in the same class (Bacillariophyceae) but in the next order, Thalassiophysales D. G. Mann. Thus it is reasonable to look for further production of DA in raphid diatoms, both planktonic and benthic.

APPENDIX 17.1

Authorities for taxonomic names of diatom genera and species used in this chapter

Amphora Ehrenberg
Amphora coffeaeformis (Agardh) Kützing
A. ovalis (Kützing) Kützing
Cerataulina H. Peragallo ex Schütt
Cerataulina pelagica (Cleve) Hendey
Chaetoceros Ehrenberg
Chaetoceros aequatorialis Cleve
C. atlanticus Cleve
C. borealis Bailey
C. castracanei Karsten
C. coarctatus Lauder
C. concavicornis Mangin
C. convolutus Castracane
C. criophilus Castracane
C. curvisetus Cleve
C. dadayi Pavillard
C. danicus Cleve
C. debilis Cleve
C. densus (Cleve) Cleve
C. dichaeta Ehrenberg
C. eibenii Grunow
C. peruvianus Brightwell
C. rostratus Lauder
C. socialis Lauder
C. tetrachaeta Ehrenberg
C. tetrastichon Cleve
Coscinodiscus Ehrenberg
Coscinodiscus argus Ehrenberg
C. centralis Ehrenberg
C. concinnus W. Smith
C. wailesii Gran et Angst
Cylindrotheca Rabenhorst
Cylindrotheca closterium (Ehrenberg) Reimann et Lewin
Guinardia H. Peragallo
Guinardia delicatula (Cleve) Hasle
Leptocylindrus Cleve
Leptocylindrus minimus Gran
Minutocellus Hasle, von Stosch et Syvertsen
Minutocellus pseudopolymorphus Hasle, von Stosch et Syvertsen
Nitzschia Hassall
Nitzschia navis-varingica Lundholm et Moestrup
N. sicula (Castracane) Hustedt
N. sigmoidea (Nitzsch) W. Smith
Pseudo-nitzschia H. Peragallo in H. et M. Peragallo

Pseudo-nitzschia australis Frenguelli
P. delicatissima (Cleve) Heiden
P. fraudulenta (Cleve) Hasle
P. heimii Manguin
P. multiseries (Hasle) Hasle
P. multistriata (Takano) Takano
P. pseudodelicatissima (Hasle) Hasle
P. pungens (Grunow ex Cleve) Hasle
P. seriata (Cleve) H. Peragallo
P. seriata f. *obtusa* (Hasle) Hasle
P. subfraudulenta (Hasle) Hasle
P. turgidula (Hustedt) Hasle
Rhizosolenia Brightwell
Rhizosolenia chunii Karsten
Skeletonema Greville
Skeletonema costatum (Greville) Cleve
Tabularia Kützing
Tabularia affinis (Kützing) Snoeijs
Thalassiosira Cleve
Thalassiosira curviseriata Takano
T. delicatula Ostenfeld in Borgert
T. diporocyclus Hasle
T. fragilis G. Fryxell
T. gravida Cleve
T. mala Takano
T. mediterranea (Schröder) Hasle
T. minuscula Krasske
T. nordenskioeldii Cleve
T. partheneia Schrader
T. proschkinae Makarova
T. subtilis (Ostenfeld) Gran
T. tubifera G. Fryxell
T. weissflogii (Grunow) G. Fryxell et Hasle

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18

Taxonomy of harmful marine raphidophytes

G. M. Hallegraeff and Y. Hara

18.1 Introduction

Small golden-brown (fucoxanthin-containing; Mostaert *et al*., 1998) flagellates within the class Raphidophyceae Chadefaud ex Silva (= class Chloromonadophyceae Papenfuss) of the division Chrysophyta can pose a serious threat to finfish aquaculture. Throndsen (1993) created the order Chattonellales for the four marine genera *Chattonella* Biecheler, *Heterosigma* Hada, *Fibrocapsa* Toriumi et Takano and *Olisthodiscus* N. Carter and tentatively also included *Oltmannsia* Schiller. The further genus *Haramonas* Horiguchi has since been added (Horiguchi, 1996). Freshwater species (not discussed here) are coloured green with pigment affinities to the Xanthophyceae (without fucoxanthin; Fiksdahl *et al*., 1984) and were classified in the order Raphidomonadales Chadefaud, family Vacuolariaceae Luther. In the 1970s and 1980s much attention focused on species of the genus *Chattonella* as the cause of mortality of cultured yellowtail and red sea bream in Japanese inland seas (Okaichi, 1983) but in the 1990s attention has shifted to *Heterosigma* as the cause of fish kills in New Zealand, Chile and British Columbia (Chang *et al*., 1990). Considerable confusion exists regarding the taxonomy of the group and various members have often been mistakenly assigned to other algal classes such as cryptomonads, chrysophytes, dinoflagellates and euglenoids.

Basic features of the cell structure include two subequal, heterodynamic flagella arising from a more or less pronounced flagellar groove (*Olisthodiscus*, *Heterosigma*): the forward flagellum bears two rows of fine tripartite hairs, while the trailing flagellum is smooth and lies close to the surface of the cell. The flagellar bases have a unique system of cross-banded, fibrous roots (Vesk and Moestrup, 1987; Inouye *et al*., 1992). The cells are naked, dorsiventrally flattened and contain numerous ejectosomes (*Heterosigma*), trichocysts and mucocysts (*Chattonella*, *Fibrocapsa*) that readily discharge, thereby rendering these species difficult subjects for microscopic studies (Vesk and Dwarte, 1980; Vesk and Puttock, 1980). For example, in 1996 a bloom of *Chattonella marina* associated with mortality of cultured bluefin tuna in South Australia had initially gone undetected due to the use of formaldehyde preserved net samples (Hallegraeff *et al*., 1998). The cells contain numerous golden-brown chloroplasts in the peripheral cytoplasm (termed ectoplasm), which upon cell disintegration gives a characteristic 'raspberry-like' appearance to the cell remains. Ultrastructural studies are now available for *Heterosigma akashiwo* (Leadbeater, 1969 [as *Olisthodiscus luteus*], Hara and Chihara, 1985), *Olisthodiscus luteus* (Hara *et al*., 1985), *Fibrocapsa japonica* (Hara and Chihara, 1985), *Chattonella subsalsa* (Mignot, 1976), *C. marina* and *C. antiqua*

(Hara and Chihara, 1982), *Chattonella globosa*, *C. minima*, *C. ovata* and *C. verruculosa* (Hara *et al*., 1994) and *Haramonas dimorpha* (Horiguchi, 1996).

The killing mechanism of raphidophyte blooms is still poorly understood. Both physical clogging of fish gills by mucus excretion as well as gill damage by haemolytic substances may be involved (Shimada *et al*., 1983; Chang *et al*., 1990). Increasing evidence is now pointing towards the production of superoxide and hydroxyl radicals as a major mechanism of fish mortality by both *Chattonella* (Tanaka *et al*., 1994) and *Heterosigma* (Yang *et al*., 1995). Onoue and Nozawa (1989) also reported high-performance liquid chromatography (HPLC) evidence for production of brevetoxin-like neurotoxins by *Chattonella*, *Heterosigma* and *Fibrocapsa* but detailed mass spectroscopic confirmation is still required. Free fatty acid production, notably of eicosapentaenoic acid (17-27% of total fatty acids), may also play a role in fish mortalities caused by raphidophyte blooms (Marshall *et al*., 2002).

18.2 HARMFUL MARINE RAPHIDOPHYTES

18.2.1 *Heterosigma akashiwo* (Hada) Hada

Basionym: *Entomosigma akashiwo* Hada 1967
Synonyms:? *Heterosigma inlandica* Hada 1968, '*Olisthodiscus luteus*' Plymouth cultures 12A and 239, *Chattonella akashiwo* (Hada) Loeblich III, *Olisthodiscus carterae* Hulburt 1965, *Heterosigma carterae* (Hulburt) Taylor 1992.

Illustrations: Hara and Chihara (1982), LM, TEM; Hara, in Fukuyo *et al*. (1990, pp. 346–7), LM, SEM, TEM; present Fig. 18.1.

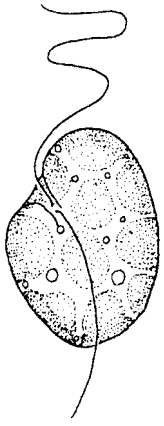

Figure 18.1
Heterosigma akashiwo.

Cell shape and size: the potato-shaped cells (8–25 × 6–15 µm) are slightly compressed dorso-ventrally and vary in shape from spheroidal to ovoid or oblong according to culture conditions and cell age.

Flagella: two subequal, heterodynamic flagella are present, an anterior pulling flagellum and a posterior rigid flagellum, both arise from an oblique groove which starts subapically and terminates half-way along the length of the cell. The cells exhibit a spiralling swimming pattern.

Chloroplasts: many (10–30) yellow-brown to brown chloroplasts are located in the periphery of the cell. A pyrenoid protrudes from the inner chloroplast surface towards the centre of the cell and is invaded by thylakoids.

Other features: The centrally located nucleus is teardrop-shaped. Eyespots or contractile vacuoles are absent. Mucocysts are present, similar to those found in *Chattonella* and *Fibrocapsa*.

Life cycle: As part of its life cycle, this species produces benthic resting cells consisting of agglutinated masses of non-motile brown cells of variable size and shape (Tomas, 1978). For a description of the cyst stage, see Chapter 20.

Distribution: Widespread in coastal and brackish waters in the Pacific and Atlantic. A common red-tide species in Japan (*akashiwo*: red tide), where it has never caused fish kills. A major problem organism for finfish aquaculture in British Columbia, Washington State, Chile, New Zealand and possibly Singapore.

Note: This species has often been confused with *Olisthodicus luteus* (see Gibbs *et al*., 1980; Leadbeater, 1969; Tomas, 1978), but can be distinguished by its cell shape, colour and swimming pattern. *H. akashiwo* is probably also conspecific with *H. inlandica* Hada 1968 (Throndsen, 1993). The proposed name change *Heterosigma carterae* (Hulburt) Taylor 1992 has been rejected because of the failure by Hulburt (1965) to designate the holotype of the name (Throndsen, 1996).

18.2.2 *Olisthodiscus luteus* N. Carter 1937

Illustrations: Hara *et al*. (1985), LM, SEM, TEM; Hara, in Fukuyo *et al*. (1990, pp. 348–9), LM, SEM, TEM; present Fig. 18.2.

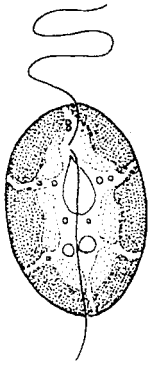

Figure 18.2
Olisthodiscus luteus.

Cell size and shape: strongly dorsiventrally compressed, 15–25 µm long, 10–16 µm wide, 5–7 µm thick.

Flagella: anterior flagellum 1–1.2 times cell length, posterior flagellum 0.8–1 times cell length, both arising from a longitudinal furrow on the mid-ventral side of the cell. The cells do not rotate but exhibit a smooth forward swimming pattern.

Chloroplasts: many (5–13) flattened, disc-shaped, pale green-yellow chloroplasts (*luteus*: yellow) are located in the cell periphery. The ventral side of the cell is devoid of chloroplasts. The pyrenoid is free from thylakoids, but is invaginated by tubular structures.

Other features: Eyespots, contractile vacuoles, mucocysts and lipid bodies are absent.

Life cycle: Siliceous statospores with a simple circular opening are formed asexually. Sexual reproduction is unknown.

Distribution: Salt marshes of Europe, North America, South Africa and Japan.

Note: *Olisthodiscus carterae* Hulburt belongs in *Heterosigma* (see Taylor, 1992), while *O. magnus* Hulburt probably belongs in *Chattonella*.

18.2.3 *Fibrocapsa japonica* Toriumi et Takano 1973

Synonym*: Chattonella japonica* (Toriumi et Takano) Loeblich III et Fine 1977, '*Exuviella* sp.' (isolate FCRG 51)

Illustrations: Hara and Chihara (1985), LM, TEM; Hara, in Fukuyo *et al*. (1990, pp. 344–5), LM, TEM; present Fig. 18.3.

Cell size and shape: slightly flattened, ovate to obovate in ventral view, 20–30 × 15–17 µm.

Flagella: the anterior flagellum is as long as the cell, and the posterior flagellum is 1.2 times the cell length. Both emerge from an anterior gullet.

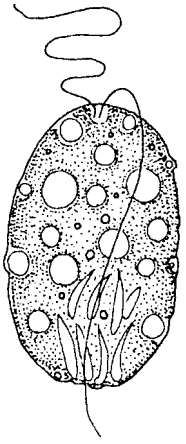

Figure 18.3
Fibrocapsa japonica.

Chloroplasts: many discoid, yellow-brown to golden-brown chloroplasts are densely packed in the cell, giving the appearance of a single reticulate chloroplast. A pyrenoid is present in each chloroplast.

Other features: Rod-shaped mucocysts in the posterior end of the cell eject long threads (up to 300 µm). The nucleus is located in the centre of the cell. Eyespots and contractile vacuoles are absent. Spherical cyst stages can be found adhering to diatom frustules (see Chapter 20).

Distribution: Coastal waters of Japan, Australia, New Zealand, California, northern Europe and Brazil.

18.2.4 *Chattonella antiqua* (Hada) Ono 1980

Basionym: *Hemieutreptia antiqua* Hada 1974

Illustrations: Hara and Chihara (1982), LM, TEM; Hara, in Fukuyo *et al*. (1990, pp. 332–3), LM, TEM; present Fig. 18.4.

Cell size: large cells, 70–130 µm long, 30–50 µm wide, with a posterior tail.

Flagella: two subequal, heterodynamic flagella emerge from the bottom of an anterior gullet.

Chloroplasts: many ellipsoid chloroplasts are radially arranged. A naked pyrenoid is located at the inner pole of the chloroplast. Several less stacked thylakoids pass through the pyrenoid matrix.

Other features: Numerous electron-dense particles are located in the cytoplasm, immediately below the cell surface. A teardrop-shaped nucleus is situated in the centre of the cell. Contractile vacuoles, eyespots or mucocysts are absent.

Life cycle: Asexual reproduction is by binary division. A diplontic life cycle has been described by Yamaguchi and Imai (1994), with cyst formation occurring after meiosis in vegetative cells. The hemispherical cysts have a simple pore on top (see Chapter 20).

Distribution: A causative organism of massive fish kills, known from Japan, Korea, the Netherlands, the United States and Brazil.

Note: The form described as *C. ovata* Hara et Chihara (Hara *et al*., 1994, Figs. 19–22, 24–25; present Fig. 18.5) may be an ecotype of *C. antiqua* and is still only known from the Japanese type locality.

18.2.5 *Chattonella marina* (Subrahmanyan) Y. Hara et Chihara 1982

Basionym: *Hornellia marina* Subrahmanyan 1954

Illustrations: Hara and Chihara (1982), LM, TEM; Hara, in Fukuyo *et al*. (1990, pp. 336–7), LM, TEM; present Fig. 18.6.

Cell size and shape: the cell, 30–70 µm long × 20–30 µm wide, is asymmetrical in lateral view, slightly flattened, oblong to obovoid in shape, with a posterior tail.

Flagella: the two subequal flagella are approximately equal to the length of the cell and emerge from the bottom of an anterior depression in the cell.

Chloroplasts: many, green to yellowish-brown, ellipsoid chloroplasts, are arranged radially. A naked pyrenoid is located on the inner pole of the chloroplast.

Other features: The teardrop-shaped nucleus is situated in the centre of the cell. Contractile vacuoles, eyespots and mucocysts are absent. Asexual reproduction is by

binary division. A diplontic life cycle has been described by Yamaguchi and Imai (1994), with cyst formation occurring after meiosis in vegetative cells. The hemispherical cysts have a simple pore on top (see Chapter 20).

Distribution: Brackish coastal areas rich in organic material from India, Australia, New Zealand, China, Korea, Japan, Brazil, USA and the Netherlands.

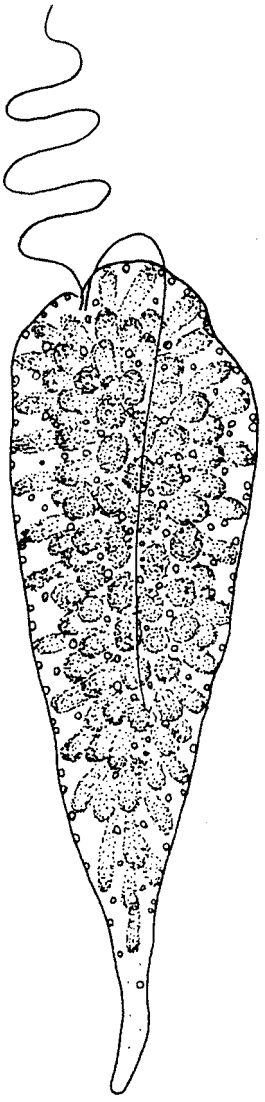

Figure 18.4
Chattonella antiqua.

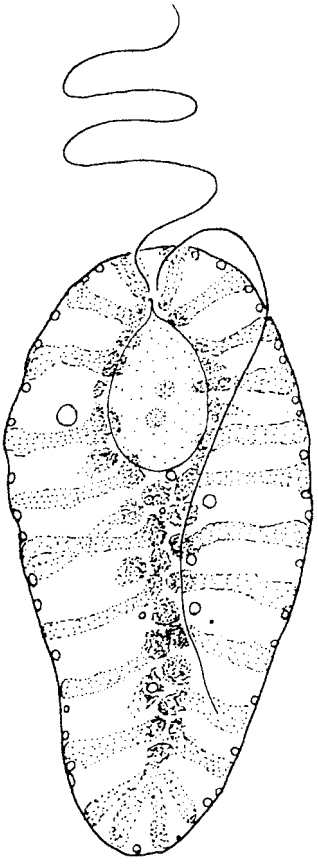

Figure 18.5
'*Chattonella ovata*' form.

Note: This species is often regarded as synonymous with *Chattonella subsalsa* Biecheler 1936 (present Fig. 18.7), but Hara and Chihara (1982) discuss reasons to keep them separate, i.e. no thylakoids penetrating the pyrenoids, and oboe-shaped mucocysts present in *C. subsalsa* but not *C. marina*. The form described as *C. minima* Hara et Chihara (Hara *et al*., 1994, Figs. 13–15, 17–18; present Fig. 18.8)

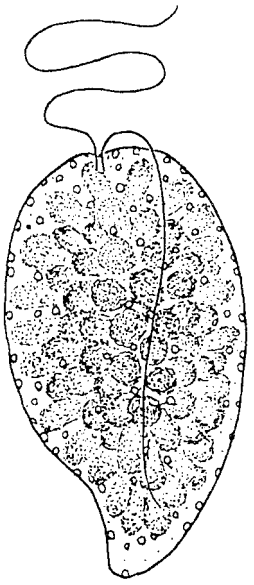

Figure 18.6
Chattonella marina.

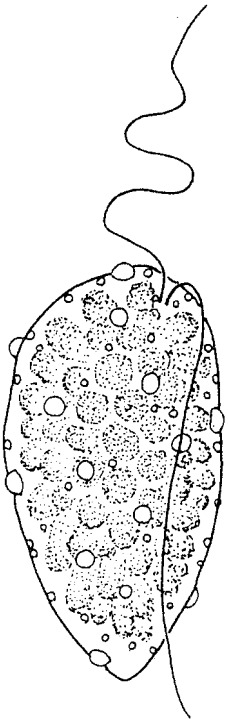

Figure 18.7
Chattonella subsalsa.

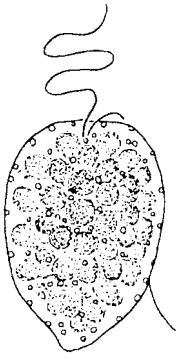

Figure 18.8
Chattonella minima' form.

may be an ecotype of *C. marina* and is still only known from the Japanese type locality. The species referred to as *Olisthodiscus magnus* Hulburt 1965 is most probably conspecific with *C. marina*, but its description is incomplete and a re-examination of material from the type locality in Woods Hole ponds is required before a conclusion can be drawn on its precise identity. The separation of *C. antiqua* and *C. marina* also requires re-examination, because it is based solely on cell size and is not supported by chemotaxonomy of pigments and lipids (Mostaert *et al*., 1998; Marshall *et al*., 2002) nor molecular sequencing (Hirashita *et al*., 2000; Sako *et al*., 2000).

18.2.6 *Chattonella subsalsa* Biecheler 1936

Illustrations: Mignot (1976), TEM, Figs. 2–19; present Fig. 18.7.

Cell size and shape: the cell, 30–50 µm long × 15–25 µm wide, is slightly flattened, and lanceolate in lateral view.

Flagella: the two subequal, heterodynamic flagella emerge from the bottom of an anterior depression in the cell.

Chloroplasts: many, green to brown, ellipsoid chloroplasts, are arranged radially within the vacuolated ectoplasm. A pyrenoid is located on the inner pole of the chloroplast, and is difficult to recognize by light microscopy. No thylakoids enter into the pyrenoid matrix.

Other features: The teardrop-shaped nucleus is situated in the centre of the cell within the cytoplasmic endoplasm. Contractile vacuoles and eyespots are absent. Many mucocysts with 'oboe'-shaped inclusions are distributed around the cell periphery. Cyst formation and sexual reproduction are unknown.

Distribution: Eutrophic coastal areas of the Mediterranean from France to Algeria.

Note: This species appears closely related to *C. marina*, but can be distinguished by its colour and protrusion of heads of mucocysts on the cell surface. Considering that this is the type species of *Chattonella*, a re-examination of Mediterranean material of *C. subsalsa* is needed in order to reach a conclusion on the distinction between the species *C. subsalsa* and *C. marina*.

18.2.7 *Chattonella globosa* Y. Hara et Chihara, in Hara *et al.* (1994)

Illustrations: Fukuyo *et al*. (1990, pp. 334–5); Hara *et al*. (1994, Figs. 1–12), LM, TEM; Hosaka *et al*. (1991), LM; also known as 'flagellate X' (Scotland, Ireland); present Fig. 18.10.

Cell size: nearly globose, 40–55 µm diameter.

Flagella: two unequal flagella emerge from the shallow depression at the cell anterior.

Chloroplasts: numerous pale-brown to golden-brown, small elliptical chloroplasts without pyrenoid are located throughout the cytoplasm.

Other features: A spherical nucleus is located in the centre of the cell. Several large mucocysts with nail-shaped inclusions are distributed along the cell periphery. No contractile vacuoles nor eyespot are present. Asexual reproduction takes place by binary fission while swimming. Cyst formation and sexual reproduction are unknown.

Distribution: Eutrophic coastal areas of Japan, South-East Asia, Australia and Canada. This species is often confused with *C. antiqua*, but can be distinguished by cellshape and characteristics of chloroplasts, flagellation and mucocysts. It is known

to cause respiratory damage to fish, similar to that caused by other *Chattonella* species.

18.2.8 *Chattonella verruculosa* Y. Hara et Chihara, in Hara *et al*. (1994)

Illustrations: Fukuyo *et al*. (1990, pp. 342–3), LM, TEM; Hara *et al*. (1994, Figs. 26–33); present Fig. 18.9.

Cell size: globose, 12–45 µm long.

Flagella: two unequal flagella emerge from the cell anterior.

Chloroplasts: numerous pale-yellow to yellow-brown, small discoid chloroplasts with a single embedded pyrenoid.

Other features: No electron-dense (osmiophilic) particles are present in the peripheral cytoplasm (as found in *C. antiqua* and *C. marina*). A spherical nucleus is located in the centre of the cell. Several large mucocysts with bullet-shaped inclusions are distributed along the cell periphery. Verrucose protrusions of their heads are visible by light microscopy.

Distribution: Only known with certainty from the Seto Inland Sea in Japan. A similar species bloomed in the North Sea/Skagerrak in April–May 1998 (Backe-Hansen *et al*., 2000), and *C. cf.verruculosa* was also reported from Delaware Inland Bays, North America (Bourdelais *et al.*, 2002).The form described as *C. globosa* Hara et Chihara (Fig 18.10) also has mucocysts with nail-shaped inclusions but verrucose appendages have not been reported.

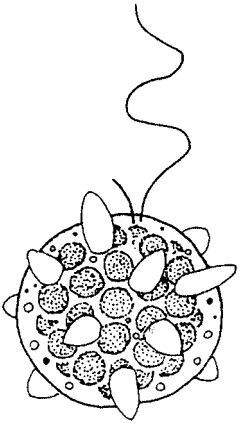

Figure 18.9
Chattonella verruculosa.

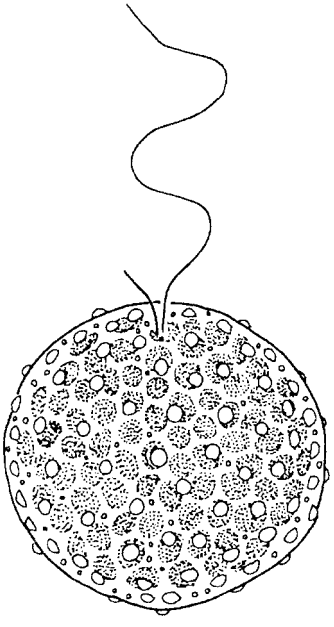

Figure 18.10
'*Chattonella globosa*' form.

18.2.9 *Haramonas dimorpha* Horiguchi 1996

Illustrations: Horiguchi (1996), Figs. 106 (LM), 8–18 (TEM); present Fig. 18.11.

Cell size: club-shaped cells, 24–39 µm long, 10–15 µm wide.

Flagella: two heterodynamic flagella emerge from the cell anterior.

Chloroplasts: numerous (10–20) disc-shaped, yellow-brown chloroplasts arranged peripherally in an overlapping manner.

Other features: A diagnostic tubular invagination occurs at the posterior end of the cell. Mucocysts are distributed along the cell periphery but trichocysts are absent. The organism occurs in two distinct life cycle stages ('*dimorpha*'), a club-shaped motile form and a spherical non-motile benthic form.

Distribution: Only known to date from the type locality in tropical mangrove waters of north-eastern Australia as well as a coral lagoon in Saipan. A similar, but not identical, organism was associated with fish kills in Sydney Harbour in 1996 (Ajani *et al.*, 2001).

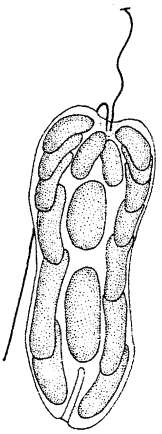

Figure 18.11
Haramonas dimorpha.

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19

Taxonomy of harmful cyanobacteria

G. Cronberg, E. J. Carpenter and W. W. Carmichael

Marine, brackish water and freshwater cyanobacteria can produce toxins which result in neuromuscular and organ distress as well as external contact irritation. These toxins can affect humans as well as terrestrial animals and aquatic life. More than 55 species, belonging to 30 genera, have been shown to be toxin producers. More toxic cyanobacterial species have been recorded from fresh than from brackish or marine water. Here we describe methods for collecting and identifying these cyanobacteria as well as a brief description of symptoms. For the species herein we have used the classification scheme by Anagnostidis and Komárek (1985, 1988, 1990) and Komárek and Anagnostidis (1986, 1989, 1999). Illustrations are originals or as presented in Geitler (1932) or Kondrateva (1968). This chapter is intended as a guide to the taxonomy and identification of toxic aquatic cyanobacteria and the most common species are illustrated. For precise identification we refer the reader to specialized publications.

19.1 Collection

Cyanobacteria can exhibit a patchy distribution in nature and, if a quantitative estimate of their abundance is desired, care should be taken to sample them adequately. Many, but not all, cyanobacteria have gas vesicles (called 'gas vacuoles' in older literature) and are buoyant, thus causing them to accumulate in blooms on the water surface or in long, narrow surface streaks associated with Langmuir circulation cells. Others can exhibit depth-keeping and have dense accumulations at specific depths. For example, *Trichodesmium* spp. typically have a depth maximum at about 15 m in tropical and subtropical waters. Thus care should be taken to sample surface waters as well as accumulations in Langmuir circulation cells and specific depths in the water column. Generally, if water bottles are used for collection, samples taken at 5 m intervals from surface to the bottom of the euphotic zone will reveal any vertical accumulations and one can visually determine sample sites in Langmuir circulation cells.

One additional precaution is to draw the sample from the water bottle as soon as possible after collection, as cyanobacteria with gas vesicles will rise to the top of the bottle and the sampling port is typically at the bottom. If water cannot be drawn immediately, then the contents of the bottle should be mixed prior to taking the sample.

19.2 PRESERVATION

Identification is preferably made on live material as solutions used to preserve can distort cells. However, if the cyanobacteria cannot be examined when fresh, preservation with fixatives is an acceptable alternative. It is important that the preservative used for cyanobacteria be acidified. This discharges gas vesicles and allows the organism to be concentrated, either by settling chambers, or by centrifugation. Without discharge of these vesicles, the cyanobacteria would not fall to the bottom of either the settling chamber or centrifuge tube, and population density would be underestimated. To prepare a stock acidified Lugol's solution, add 200 g KI, 100 g I_2, 2,000 ml H_2O, and 190 ml glacial acetic acid. About 10 drops of this solution should be added to 200 ml of sample water to give the colour of strong tea. Sample bottles should be tightly capped, placed in the dark, and preferably kept in a cool place. If the colour of the sample water begins to fade, add more Lugol's solution and check that the caps are tight. As long as the colour remains in the sample, these will be preserved indefinitely.

An acidified formalin (*c*. 40% formaldehyde) solution can be made by mixing equal parts of formalin and concentrated acetic acid. For preservation, add 2 ml of this acidified solution to a 100 ml sample (giving a 0.4% formaldehyde solution). If tightly capped, these samples will be stable.

19.3 IDENTIFICATION AND QUANTIFICATION

Noxious species can be benthic or planktonic and can also exist as cells, free trichomes or in the colonial state. For the larger filamentous cyanobacteria, identification can be made using a microscope at 400× magnification with the sample placed on a standard glass slide with a cover slip. However, for quantitative counts it is important that the method of counting be adapted to the size and density of the organism, as the range in size from the smallest toxic cyanobacterium to the largest is about three orders of magnitude. There are many acceptable methods for identifying and quantifying cyanobacterial populations (see Chapter 4). Generally, a Sedgewick-Rafter chamber (20 mm × 50 mm × 1 mm deep) can be used for quantitative counts. A relatively inexpensive plastic Sedgewick-Rafter cell is available from Electron Microscope Sciences, Box 251, Fort Washington, PA 19034, USA. This chamber holds 1 ml of sample, and the investigator can either put a concentrated (*c*. 10 ml or 15 ml concentrated with a centrifuge) or unconcentrated sample in the chamber. Transects are then made, and as long as the width of the field-of-view is known, the density of different species present can be calculated. One disadvantage of the Sedgewick-Rafter cell is that magnifications of 400× cannot be used with a standard 40× objective. Long-working-distance 40× objectives can be purchased. The Palmer-Maloney counting chambers are shallower, with a depth of 0.4 mm, and a standard 40× objective can be used. This chamber is 17.9 mm diameter and it holds 250 mm^2. All but the smallest cyanobacteria can be identified in this chamber, but because of its limited volume, it is generally necessary to concentrate the sample prior to filling the chamber. Methods for isolating and culturing cyanobacteria are given by Stein (1973).

Toxic strains of the coccoid picocyanobacterium *Synechococcus* cannot be identified to the species level using light microscopy. Identification of these

picoplankters to genus is typically done using epifluorescence microscopy, and plankton samples are mounted on membrane filters. Formalin-killed samples should be used for epifluorescence counts and stored in a cool (preferably refrigerated), dark place. As *Synechococcus* spp. are very abundant and widespread and, as toxic and non-toxic strains cannot yet be distinguished, it would be difficult to associate this organism with a toxic event. In this case it would be prudent to attempt to culture these coccoid cyanobacteria and carry out toxicity assays on isolates.

19.4 Taxonomy of cyanobacteria

The cyanobacteria (often called blue-green algae) are very diverse and widespread phototrophic prokaryotes. In the modern taxonomic literature they are called cyanoprokaryotes (Komárek and Anagnostidis, 1998). According to different morphological characters, the cyanobacteria are divided into four orders, Chroococcales, Oscillatoriales, Nostocales and Stigonematales (Anagnostidis and Komárek, 1985, 1988, 1990; Komárek and Anagnostidis, 1986, 1989).

- Chroococcales includes unicellular or colony-forming cyanobacteria, which do not form true filaments with direct physical contact between cells. However, in more complex types polarized thallus and differentiated cells can appear. Cell division takes place in one, two or more planes.
- Oscillatoriales includes filamentous forms, which have cell division in only one plane, perpendicular to the length axis of the filament. They do not develop heterocytes (nitrogen-fixing cells) [called 'heterocysts' in older literature] or akinetes (resting cells, spores).
- Nostocales also includes filamentous forms with cell division in only one plane, perpendicular to the length axis. They develop heterocytes and akinetes. False branching can appear but never true branching.
- Stigonematales are filamentous forms with heterocytes, akinetes and true branching.

Table 19.1 lists most of the toxic species found to date, which toxin they produce and their habitat. However, the cyanobacteria have mainly been studied in the temperate zone and few tropical species have been investigated. Here we describe the morphology only as seen in the light microscope, but in the future a more multidisciplinary approach to taxonomy will be required.

19.4.1 Toxic cyanobacteria

19.4.1.1 *Chroococcales*

***Coelosphaerium* Nägeli 1849**

Coelosphaerium is mainly a planktonic freshwater genus with worldwide distribution. It comprises about 15 species (Komárek and Anagnostidis, 1998). Only one species, *C. kuetzingianum* Nägeli, has been reported to have toxic effects (Fitch *et al.*, 1934). *C. kuetzingianum* has free-floating, more or less round colonies up to about 100 µm in diameter. Cells are spherical, arranged in one or two layers at the colony surface or just under the surface of the homogeneous colourless mucilage (Fig. 19.1B), and are green to olive-green, granular, 1.8–3 µm, without aerotopes (gas vesicles). *Coelosphaerium* appears in mesotrophic water-bodies and probably has a cosmopolitan distribution, but is rather uncommon.

TABLE 19.1 Toxic cyanobacteria from marine, brackish water and freshwater

Species	Toxin or toxic effect	Habitat	References
Chroococcales			
Coelosphaerium kuetzingianum Nägeli	Neuro/hepato toxins, mouse test	Freshwater	Fitch *et al.* (1934)
Cyanobium bacillare (Butcher) Komárek; syn. *Synechococcus bacillare* Butcher	Microcystins	Brackish water	This chapter
Microcystis aeruginosa Kützing	Microcystins	Freshwater	Carmichael *et al.* (1988)
Microcystis botrys Teiling	Microcystins	Freshwater	Henriksen (1996)
Microcystis viridis (A. Braun in Rabenhorst) Lemmermann	Microcystins	Freshwater	Watanabe *et al.* (1988)
Microcystis wesenbergii Komárek	Microcystins	Freshwater	Yasuno *et al.* (1995)
Snowella lacustris (Chodat) Komárek; syn. *Gomphosphaeria lacustris* Chodat	Hepato toxins, mouse test	Freshwater	Gorham and Carmichael (1988)
Synechococcus sp. Nägeli	Haemolytic toxin	Brackish/marine water	Mitsui *et al.* (1987)
Synechocystis sp. Sauvageau	Microcystins	Waste-water pond	Oudra *et al.* (1998); Odebrecht *et al.* (in press)
Woronichinia naegeliana (Unger) Elenkin; syn. *Gomphosphaeria naegeliana* (Unger) Lemmermann	Neuro/hepato toxins, mouse test	Freshwater	Berg *et al.* (1986)
Oscillatoriales			
Limnothrix redekei van Goor	Microcystins	Freshwater	Gkelis *et al.* (2001)
Lyngbya majascula Harvey	Neurotoxins, lyngbyatoxin-a, debromoaplysiatoxin	Marine water/tropical	Osborne *et al.* (2001)
Lyngbya wollei (Farlow ex Gomont) Carmichael *et al.*	PSP toxins	Freshwater	Carmichael *et al.* (1988); Carmichael *et al.* (1997)

…/…

TABLE 19.1 *(Suite)*

Species	Toxin or toxic effect	Habitat	References
Oscillatoriales			
Oscillatoria acutissima Kufferath	Dermatoxin	Freshwater	Barchi *et al*. (1983)
Oscillatoria nigro-viridis Thwaites	Debromoaplysiatoxin, oscillatoxin-a	Marine water	Mynderse *et al*. (1977)
Planktothrix agardhii (Gomont) Anagnostidis et Komárek; syn. *Oscillatoria agardhii Gomont*	Microcystins, anatoxin	Freshwater	Sivonen *et al*. (1989)
Planktothrix mougeotii (Kützing ex Geitler) Anagnostidis et Komárek; syn. *Oscillatoria mougeotii* Kützing ex Geitler; *Oscillatoria agardhii* var. *isothrix* Skuja	Microcystins, anatoxin	Freshwater	Carmichael (1988)
Planktothrix perornata (Skuja) Anagnostidis et Komárek; syn. *Oscillatoria perornata* Skuja	Microcystins	Freshwater/tropical	Nogueira and Vasconcelos (2001)
Planktothrix planctonica (Elenkin) Anagnostidis et Komárek; syn. *Oscillatoria ornata* f. *planctonica* Elenkin	Microcystins	Freshwater	Nogueira and Vasconcelos (2001)
Planktothrix rubescens (DeCandolle ex Gomont) Anagnostidis et Komárek; syn. *Oscillatoria rubescens* DeCandolle	Microcystins	Freshwater	Skulberg and Skulberg (1985); Sivonen *et al*. (1989); Ericsson (1990)
Phormidium formosum (Bory) Komárek et Anagnostidis; syn *Oscillatoria formosa* Bory	Microcystins, homoanatoxin-a	Freshwater	Skulberg *et al*. (1992); Steffensen *et al*. (2001)
Pseudanabaena catenata Lauterborn	Anatoxin	Freshwater	Gorham *et al*. (1982)
Trichodesmium erythraeum (Ehrenberg) Gomont	Neurotoxins, microcystins	Marine water	Hawser and Codd (1992); Shaw *et al*. (2001); Janson *et al*. (1995)
Trichodesmium thiebautii Gomont ex Gomont	Neurotoxins, microcystins	Marine water	Hawser and Codd (1992); Shaw *et al*. (2001); Janson *et al*. (1995)

…/…

TABLE 19.1 *(Suite)*

Species	Toxin or toxic effect	Habitat	References
Nostocales			
Anabaena bergii Ostenfeld	Cylindrospermopsin	Brackish water	Ostenfeld (1908); Couté and Preisig (1978)
Anabaena circinalis Rabenhorst; syn. *Anabaena hassalii* (Kützing) Wittrock	Anatoxins, PSP toxins	Freshwater	Sivonen *et al.* (1989); Humpage *et al.* (1994)
Anabaena flos-aquae (Lyngbye) Brébisson	Anatoxin-a	Freshwater	Carmichael and Mahmood (1984)
Anabaena macrospora Klebahn	Anatoxin-a	Freshwater	Park *et al*. (1993)
Anabaena mendotae Treleasi	Anatoxin-a	Freshwater	Rapala *et al*. (1993)
Anabaena lemmermannii P. Richter	Anatoxin-a(s)	Freshwater	Henriksen (1996)
Anabaena planctonica Brunnthaler	Anatoxin-a	Freshwater	Park *et al*. (1993)
Anabaena spiroides Klebahn; syn. *Anabaena spiroides* var. *contracta* Klebahn	Anatoxin-a	Freshwater	Park *et al*. (1993)
Anabaena variabilis Kützing	Anatoxin	Freshwater/marine water	Andrijuk *et al*. (1975)
Anabaenopsis milleri Woronichin	Anatoxin	Freshwater	Lanaras *et al.* (1989)
Aphanizomenon gracile Lemmermann	PSP toxins	Brackish/freshwater	Pereira *et al.* (2001)
Aphanizomenon flos-aquae (Linneus) Ralfs	Anatoxin, PSP toxin	Freshwater	Rapala *et al*. (1993); Ikawa *et al.* (1982)
Aphanizmenon ovalisporum Forti	Cylindrospermopsin	Freshwater	Banker *et al.* (1997)
Cylindrospermum sp. Kützing	Neurotoxins	Freshwater	Sivonen *et al*. (1989)
Cylindrospermopsis raciborskii (Woloszynska) Seenaya et Subba Raju	Cylindrospermopsin	Freshwater	Hawkins *et al.* (1985)

…/…

TABLE 19.1 *(Suite)*

Species	Toxin or toxic effect	Habitat	References
Nostocales			
Gloeothrichia echinulata (J. E. Smith) P. Richter	Neuro/hepato toxins	Freshwater	Ingram and Prescott (1954)
Hormothamnion enteromorphoides Grunow	Hepato toxins	Marine/tropical water	Gerwick *et al*. (1986)
Nodularia spumigena Mertens	Nodularin	Brackish water	Runnegar *et al*. (1988); Carmichael *et al*. (1988)
Nostoc linckia (Roth) Bornet et Flahault	Microcystins	Brackish/freshwater	Ransom *et al*. (1978)
Nostoc paludosum Kützing	Microcystins	Freshwater	Andrijuk *et al*. (1975)
Nostoc rivulare Kützing	Microcystins	Freshwater	Davidson (1959)
Schizothrix calcicola (Agardh) Gomont	Contact dermatitis	Freshwater	Mynderse (1977)
Nostoc zetterstedtii Areschoug	Microcystins	Freshwater	Mills and Wyatt (1974)
Nostoc sp.	Microcystins	Freshwater	Beattie *et al*. (1998)
Nostoc sp.	Microcystins	Freshwater	Sivonen *et al*. (1989, 1990)
Stigonematales			
Fischerella epiphytica Ghose	Neuro/hepato toxic	Eptiphytic/tropical	Ransom *et al*. (1978)
Hapalosiphon hibernicus W. et G. S. West	Microcystins	Freshwater	Prinsep *et al.* (1992)
Hapalosiphon fontinalis (Agardh) Bornet	Neuro/hepato toxic	Freshwater	Moore (1984)
Scytonema mirabile (Dillwyn) Bornet	Antimicrobial	Freshwater	Dow and Swoboda (2000)
Scytonema ocellatum Lyngbye ex Bornet et Flahault	Scytophycin	Epiphytic	Patterson and Bolis (1984)
Scytonema pseudohofmannii Bharadwaja	Scytophycin	Epiphitic/tropical	Moore *et al*. (1986); Carmichael *et al*. (1990)
Tolypothrix byssoidea (Hassall) Kirchner	Cytotoxin	Epiphytic/cosmopolitan	Barchi *et al*. (1983)
Umezakia natans Watanabe	Cylindrospermopsin	Freshwater	Watanabe (1987); Harada *et al*. (1994)

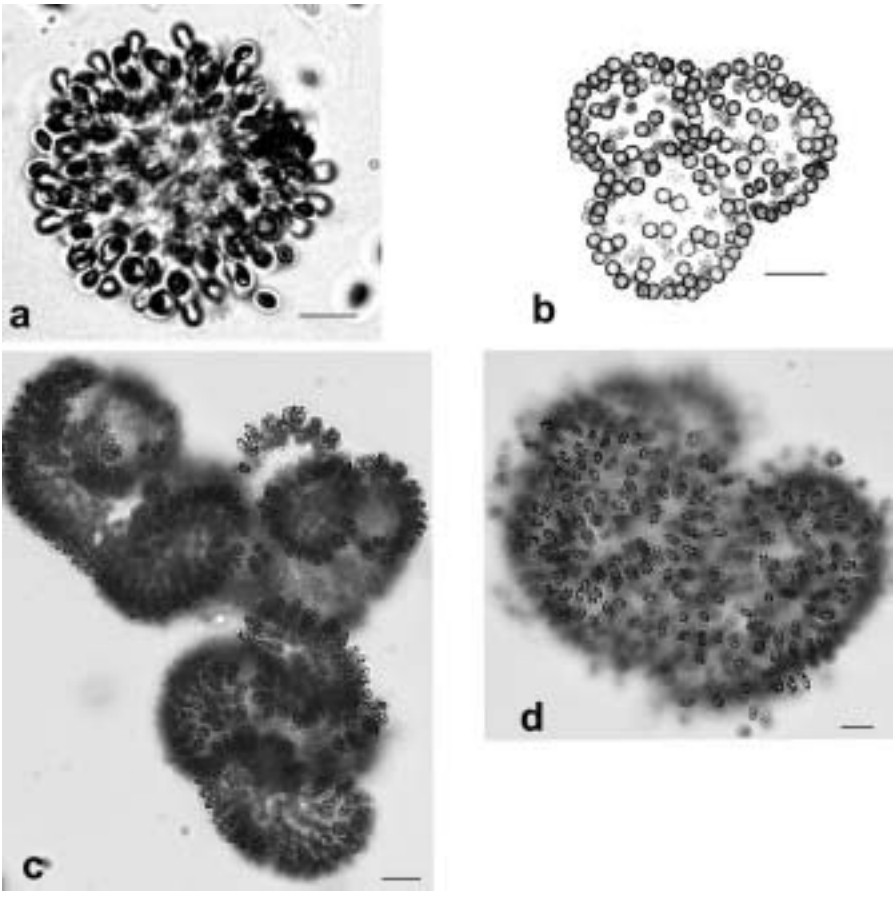

Figure 19.1
A, *Snowella lacustris*; B, *Coelosphaerium kuetzingianum* (from Skuja, 1956); C, D, *Woronichinia naegeliana*. Scale bar = 10 µm.

Cyanobium Rippka et Cohen-Bazire 1983, *Synechococcus* Nägeli 1849 and *Synechocystis* Sauvageau 1892

The *Cyanobium* group is more or less a collective assemblage for small-celled, unicellular, spherical to rod-shaped cyanobacteria, which have binary fission in one plane only. Many species that previously belonged to the genus *Synechococcus* are now included in the genus *Cyanobium* (Komárek and Anagnostidis, 1998). Several *Cyanobium* species (= *Synechococcus*) have been recorded as being hepatotoxic from both marine and brackish habitats (Mitsui *et al*., 1987) and have caused fish kill.

The genus *Cyanobium*, comprising about 10 species, is widely distributed in marine, brackish water and freshwater. *Cyanobium* species are characterized by small, ovoid to elongated cells, which divide in one plane and often appear in pairs, rarely in small pseudofilaments. Many members of the genus belong to the picoplankton.

The cells of *Cyanobium bacillare* (Butcher) (Komárek *et al*., 1999) are solitary, divide in one plane and can appear in short chains, pseudofilaments. Cells are spherical, oval to cylindrical, light blue in colour without aerotopes, dimensions 1.7–4.5 µm × 1.5 µm. In July 1995 this alga made a dense water-bloom in a park pond in the town of Malmö, Sweden. *C. bacillare* appeared as a monoculture in the pond and transparency was only about 10 cm. During the bloom period several hundreds of waterfowl such as ducks, swans and mallards were killed. The water was tested for algal toxins with high-performance liquid chromatography (HPLC) and during the bloom 86 µg/l microcystins was measured. This is the first report concerning a bird kill in connection with a toxic bloom of *Cyanobium bacillare*.

The genus *Synechocystis* is characterized by small, solitary, blue-green in colour, spherical cells. Cell division takes place in two perpendicular planes in successive generations. The cells are not in colonies, but appear singly or in pairs just after cell division. Lincoln and Carmichael (1981) investigated *Synechocystis* sp. from a waste-water pond and found that it produced anatoxin-c. Later Oudra *et al*. (1998) isolated a *Synechocystis* strain, also from a waste-water pond, however their strain contained microcystins. *Synechocystis aquatilis* caused fish kills by hepatotoxins in lagoons close to Rio de Janeiro (Odebrecht *et al*., 2002).

***Microcystis* Kützing ex Lemmermann 1907 nom. cons.**

Microcystis is a freshwater genus, which occasionally occurs in brackish water. The genus is comprised of some 10 species, according to Komárek and Anagnostidis (1998). Species in this genus are the most studied and most widely distributed geographically among the toxigenic cyanobacteria. The most common toxic species within this genus are *M. aeruginosa, M. botrys, M. flos-aquae, M. viridis* and *M. wesenbergii* (Table 19.2). Species in this genus appear to produce several peptide toxins, known as microcystins, which usually cause liver damage as well as having tumour-promoting activity.

The colonies of *Microcystis aeruginosa* can be spherical, ellipsoidal, or irregularly lobed (Figs. 19.2A, B). The clathrate colonies can be up to 8 mm in diameter. Within the colony cells are spherical, about 4–6 µm (range *c*. 3–6 µm) in diameter. Cells are surrounded by 5–8 µm wide mucilage and have many aerotopes (gas vesicles), which results in a granular appearance. The slime edge of the colony is diffuse and narrow. *M. aeruginosa* appears in plankton in eutrophic fresh and brackish water, often forming massive water-blooms.

Microcystis botrys has more or less spherical colonies with irregularly, densely, aggregated cells in mucilaginous subgroups (Fig. 19.2C). The mucilage around the colonies is wide, colourless and sometimes structured with gelatinous tubes radiating from the edge of the densely packed subcolonies. Cells are spherical with many aerotopes, which are 5–6 µm wide. *M. botrys* has more restricted distribution than *M. aeruginosa*, but is still a cosmopolitan species.

Microcystis flos-aquae has mainly microscopic spherical, compact colonies (Fig. 19.2E). The mucilaginous envelopes hardly exceed the aggregated cells in the colony. Cells are (2.5)3.5–4.8 µm wide and contain many aerotopes. *M. flos-aquae* appears in the plankton with other cyanobacteria in mesotrophic to slightly eutrophic water bodies. It is common in temperate regions.

Microcystis viridis forms microscopic, free-floating, packet-like colonies, round to cubic-formed with colourless mucilage. The slime edge is undulating and more or less follows the cells in the colonies. Cells are spherical with many

TABLE 19.2 Characteristic features of *Microcystis* species

Species	Cell size (µm)	Aerotopes	Mucilage	Colonies, size (µm), shape	Comments
M. *aeruginosa**	4–6	+	Diffluent	Colonies often clathrate, large, 600–900 µm long, cells in old colonies loosely spread	Brackish water and freshwater, frequent, forms heavy water-blooms
*M. botrys**	5–6	+	Radial, indistinct tube-like formations	Large, with subcolonies, outer cells arranged in more or less radial rows	Frequent in water-blooms, probably often misinterpreted as *Microcystis aeruginosa*
*M. flos-aquae**	(2.5) 3.5-4.8	+	Very narrow	Densely packed cells	Often associated with, and misidentified as, *Microcystis aeruginosa*
*M. ichthyoblabe**	2–3.2	+	Wide, fine, diffluent	Spherical, ellipsoidal, composed of smaller colonies, easily broken up	Uncommon, causing fish kill
M. natans	1.5–(2.23)	+	Diffluent, fine	40–200 µm, spherical to oval	Frequent, probably overlooked
*M. viridis**	3.5–7(7.9)	+	Well defined, waved, more or less refractive	Small, packet-like colonies	Occurs often with other *Microcystis species*
*M. wesenbergii**	4–7-(8.5)	+	Well defined, smooth, distinctly refractive	Ellipsoidal to irregular, sack-like	Forms sometimes monospecific water-blooms

* Toxic or harmful species.
Source: Komárek and Anagnostidis (1999).

aerotopes, mainly 3.5–7(7.9) µm wide (Fig. 19.2F). The cells can appear in two size classes. *M. viridis* is common in eutrophic waters, appears sometimes in dense water-blooms, and has a cosmopolitan distribution.

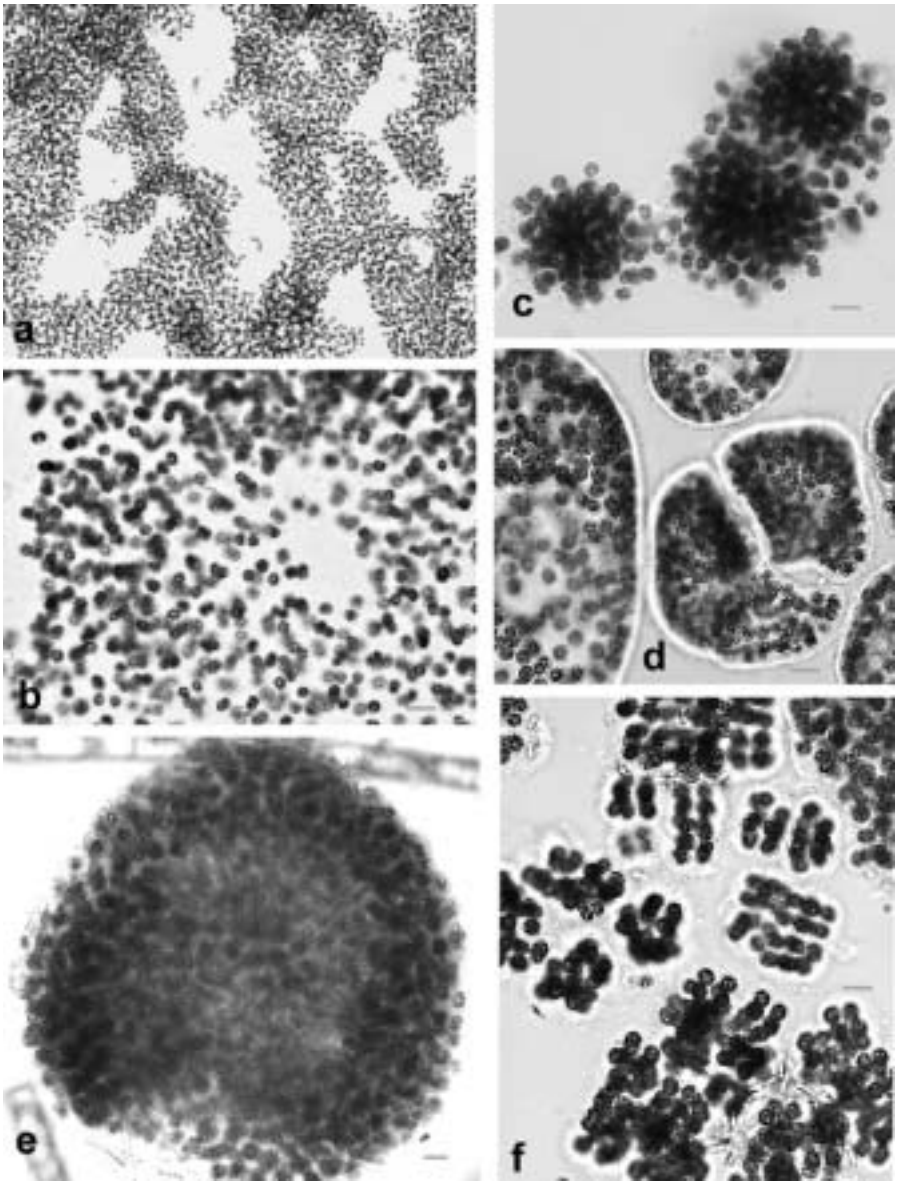

Figure 19.2
A, B, *Microcystis aeruginosa*; C, *M. botrys*; D, *M. wesenbergii*; E, *M. flos-aquae*; F, *M. viridis*. Scale bar = 10 µm.

Microcystis wesenbergii has spherical to elongate, often lobed and clathrate colonies, sometimes composed of subcolonies with a distinct, refractive mucilage edge (Fig. 19.2D). The colonies can be large up 6 mm long. Cells are more or less evenly spread in the colony, spherical and 4–7(8.5) µm in diameter, with many aerotopes. *M. wesenbergii* is common in plankton among other cyanobacteria, in eutrophic and polluted lakes and ponds, and has a cosmopolitan distribution.

***Snowella* Elenkin 1938 and *Woronichinia* Elenkin 1933**

The genus *Snowella* comprises six species and the genus *Woronichinia* 11 species (Komárek and Anagnostidis, 1998). However, several of these species were previously included in the genera *Gomphosphaeria* or *Coelosphaerium*. In 1988 the *Gomphosphaeria* complex was revised by Komárek and Hindák, and the species were split into the four genera *Coelosphaerium*, *Gomphosphaeria*, *Snowella* and *Woronichinia* according to colony structure and mode of reproduction. To date, only the species *Snowella lacustris* and *Woronichinia naegeliana* have been recorded as potential toxic species, both with hepatotoxic effects (Berg *et al*., 1986; Gorham and Carmichael, 1988).

Snowella lacustris is a planktonic species with more or less spherical or slightly irregular colonies up to about 80 µm in diameter. Cells are grey-blue-green in colour, without aerotopes, are ovoid-shaped and attached to branched mucilaginous stalks, which are oriented towards the centre of the colony (Fig. 19.1A). Cell dimensions are 2–4 µm × 1.5–3.5 µm. *S. lacustris* appears in lakes and ponds, mostly in the temperate zone but has a cosmopolitan distribution.

Woronichinia naegeliana is one of the most common planktonic cyanobacteria in the temperate region. The colonies are microscopic, spherical to kidney-shaped, sometimes lobed with colony size up to 180 µm. They are composed of radially arranged cells attached to unbranched mucilaginous stalks oriented towards the centre of the colony. Cells are blue-green, ellipsoid to ovoid in form and contain many aerotopes, dimensions 5–7 µm × 2.5–3.5(5) µm (Figs. 19.1C, D). *W. naegeliana* is common in eutrophic to mesotrophic lakes and ponds and sometimes forms waterblooms.

19.4.1.2 *Oscillatoriales*

Members of Oscillatoriales are characterized by simple filaments without specialized cells as heterocytes or akinetes and without true branching of trichomes. Some genera have the trichomes surrounded by sheaths, which can be differentially structured and coloured. The species belonging to this order are often difficult to identify because they lack characteristic features. In this chapter we describe the toxic or harmful species recorded to date. The order Oscillatoriales was carefully revised by Anagnostidis and Komárek (1988) and their systematics is followed. The characteristic features for different genera and species are presented.

***Limnothrix* Meffert 1988**

The genus *Limnothrix* comprises about 20 species, which are mostly recorded from the benthos. Only a few belong to the true plankton. The trichomes are straight or slightly bent, not constricted at the cross walls, 1–6 µm wide. Cells are long, cylindrical, containing large, centric or polar aerotopes. The filaments have no firm sheath, but sometimes a thin diffluent sheath can be observed.

Recently, the species *Limnothrix redekei* was found to be toxic and contain microcystins (Gkelis *et al*., 2001). The trichomes are grey-blue-green in colour, thin,

with cell width 1.2–2.5(3.5) µm and cell length 6–14 µm. Cells have large aerotopes at the cross walls (Fig. 19.3H). *L. redekei* is common in mesotrophic to eutrophic dams and smaller lakes.

Lyngbya C. Agardh ex Gomont 1892

The genus *Lyngbya* has straight or bent filaments and appears mostly in benthic or periphytic mats, rarely as solitary trichomes in plankton. It comprises about 70 species. The trichomes are cylindrical, enclosed in a sheath, with cell width always larger than the cell length. Cell width varies between 6 µm and 60 µm.*Lyngbya* multiplies through fragmentation or hormogonia (trichome fragmentation into small motile parts comprising up to 50 cells).

Lyngbya majuscula contains lyngbyatoxin A and debromoaplysiatoxin. Direct external contact causes 'swimmer's itch', which results in irritation of human skin. Generally *L. majuscula* grows attached to rocks or on sediments, but it can tear loose and drift for long distances. The toxins can also be concentrated by some marine invertebrates, which graze on the cyanobacterium. It has been reported from the Pacific and Atlantic Oceans, and has been a regular nuisance in Moreton Bay outside Brisbane, Australia. *L. majuscula* is distributed from tropical to temperate locations.

Trichomes of *L. majuscula* are straight and long and enclosed in a distinct sheath (Fig. 19.3G). Sheaths are unbranched. Cells are about 12 µm in diameter and only 1–1.5 µm long. The sheath is 4 µm thick, thus giving a total thickness for the filament of about 20 µm.

Lyngbya wollei (Fig. 19.9A) primarily grows in dense mats on stones and aquatic plants in running and/or stagnant water-bodies. It can be torn from the substrate and become free-floating. Filaments are straight or differently stunted in growth, forming floating black to yellow-green aggregates. The trichomes have colourless or yellowish sheaths, sometimes with false branching. The cell width is 28–47 µm and length 4–9 µm, with no constrictions at the cross walls. Carmichael*et al*. (1997) documented that *L. wollei* could produce PSP toxins

Oscillatoria Vaucher ex Gomont 1892

Oscillatoria is a prominent genus of filamentous unbranched cyanobacteria without surrounding sheaths. The filaments show oscillating or gliding moments. Several species are known for their production of cytotoxins, one of the most prominent being *Oscillatoria nigro-viridis*, which produces the contact irritant debromaplysiatoxin and oscillatoxin-a. This is a cosmopolitan species, found along the coast attached to rocks, macroalgae and sediments. The trichomes are olive-green in colour. Cells are constricted at the cross walls, 7–11 µm wide and 3–5 µm long (Fig. 19.9D). The trichomes taper at the tips and terminate in a cone-shaped apical cell.

Oscillatoria acutissima produces a dermotoxin. Cells are 1.5–2 µm in diameter, 1.5–3 times longer than wide, blue-green in colour and without calyptra. The trichomes are not constricted at the cross walls, but have long, tapering end cells (Fig. 19.9B). The filaments aggregate in layers. *O. acutissima* has been recorded from rivers and lakes.

Anagnostidis and Komárek (1988) separated the planktonic species from the genus *Oscillatoria* and incorporated them in the new genus *Planktothrix* instead. Several *Planktothrix* species from fresh and brackish water have been found to be toxic. The most common are *Planktothrix agardhii*, *P. mougeotii* and *P. rubescens*.

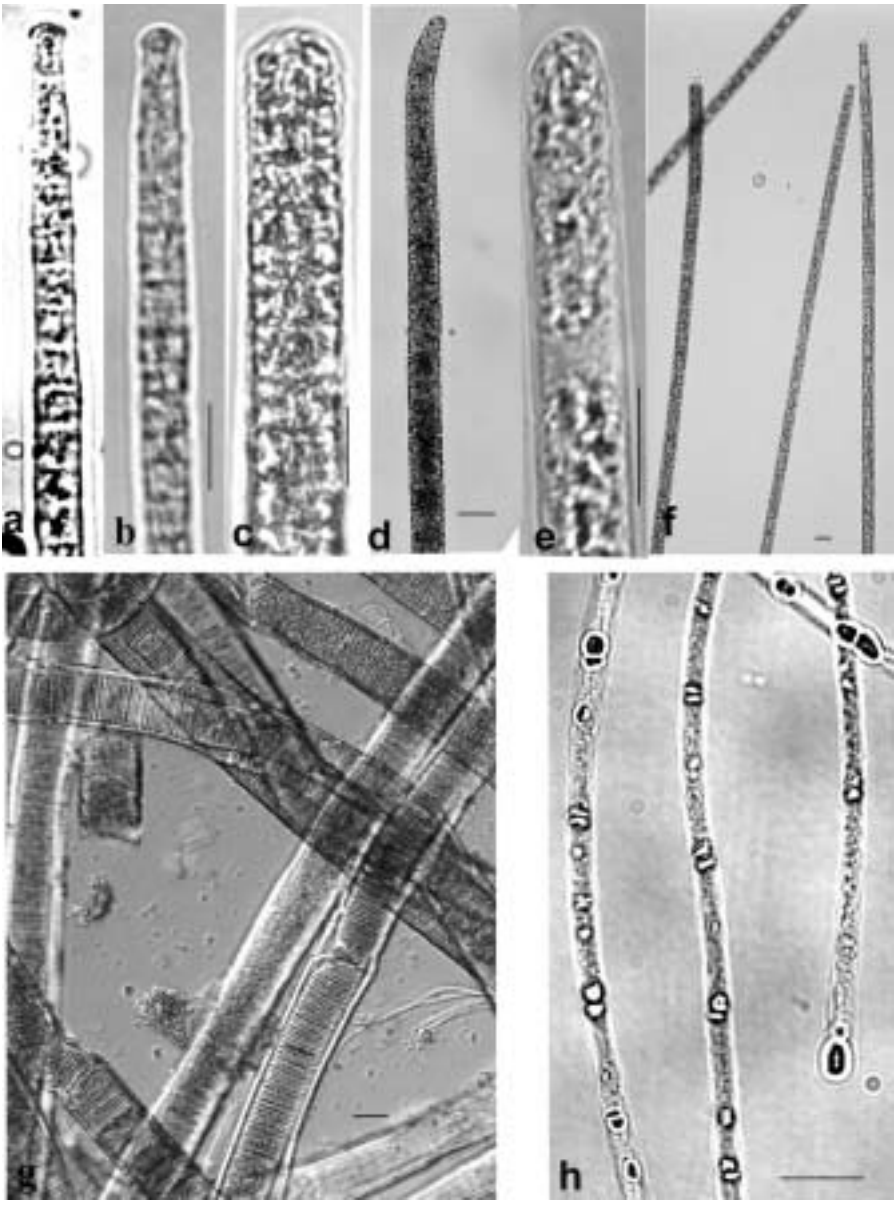

Figure 19.3
A, B, *Planktothrix agardhii*; C, *P. mougeotii*; D, *P. perornata* f. *attenuata*; E, F, *P. rubescens*; G, *Lyngbya majuscula*; H, *Limnothrix redekei*. Scale bar = 10 µm.
Sources: D, Peter Baker; F, Hans Preisig.

They produce neurotoxins anatoxin-a and cyclic peptide microcystins. *Planktothrix* species have unbranched filaments without sheaths. The trichomes vary in size, but are generally 2–10 µm in diameter and without heterocytes or akinetes.

***Planktothrix* Anagnostidis et Komárek 1988**

In *Planktothrix agardhii* and *P. rubescens* the trichomes are straight or slightly bent. Ends of trichomes can be polymorphic, and their shape can range from a pointed calyptra to a blunt end. The cells are usually longer than they are wide. *P. agardhii* is blue-green in colour with cells *c*. 4–6 µm wide (Figs. 19.3A, B), while *P. rubescens* is red with cells 4–9 µm wide (Figs. 19.3E, F). Several different forms exist within the *P. rubescens* group, producing different types of toxin. *P. mougeotii* is similar to *P. agardhii*, but always has rounded end cells without calyptra. Cell width is 5.5–10 µm (Fig. 19.3C).

Planktothrix planctonica appears in macrosopic black-blue-green layers at the bottom of stagnant waters, known from Europe and North America. The trichomes are twisted at the ends and have constrictions at the cross walls, 9–11 µm wide. The cells are much shorter than they are wide, 2–5 µm long and granulated at the cross walls. End cells are rounded without thick membranes (Fig. 19.9E). *P. planctonica* produces microcystins (Nogueira and Vasconcelos, 2001).

Planktothrix perornata was originally found in samples from Burma (Myanmar) and described by Skuja (1949). It grows in black-green mats, which can be lifted up and thus can occur free-floating in plankton, sometimes together with other cyanophytes. The trichomes are solitary or in masses, straight or slightly curved, uniformly wide and briefly attenuated at the apex. The trichomes are constricted at the cross walls, 13–15 µm wide and 2.5–6.5 µm long. When aerotopes are present the colour is black-green, when absent it is olive-green. The terminal cell is conically formed, not capitate and without calyptra. *P. perornata* was isolated (Nougueira and Vasconcelos, 2001) and found to produce small amounts of microcystins. *P. planktonica* f. *attenuata* (Skuja, 1949) is common in Australian water-bodies (Baker, 1992) and is known for producing very disagreeable odours (Fig. 19.3D).

***Phormidium* Kützing ex Gomont 1892**

The genus *Phormidium* comprises more than 170 species from different biotopes. It is mostly benthic or periphytic species growing in flat macroscopic mats. The trichomes are most often enclosed in a distinct sheath, which, however, can be missing. The trichome width is 2.5–11 µm, more or less isodiametric.

Phormidium formosum trichomes are straight, the ends slightly bent, and the cells 4–6 µm wide (Fig. 19.9C). Cells can range from quadratic in shape to about half as long as they are wide. The end cell has no calyptra. It produces microcystins and homoantoxin-a (Skulberg *et al*., 1992).

***Pseudanabaena* Lauterborn 1915**

The genus *Pseudanabaena* comprises more than 30 species. These are present in plankton as well as in the benthos. The filaments are straight or slightly bent, without sheaths. The trichomes may have constrictions at the cross walls. The cells seem as though they are connected through slime bridges, however this phenomenon is really caused by a specific procedure of cell division. Cells narrow at the cross walls. Width of trichomes is 0.8–2.5 µm. They sometimes have terminal aerotopes.

The species *Pseudanabaena catenata* has been shown to produce the algal toxin anatoxin (Gorham *et al*., 1982). Filaments are free-floating or growing in

layers in the benthos. The trichomes are straight or slightly bent with constrictions at the cross walls, light blue-green, with cell width 2–2.2 µm. Cells are long cylindrical, 1.5–8 µm long with rounded end cells (Fig. 19.5A).

***Trichodesmium* Ehrenberg 1830 ex Gomont 1892**

The genus *Trichodesmium* is similar to the genus *Planktothrix*, but is distinguished by production of mucilage, in forming colonies and also the ability to fix nitrogen. *Trichodesmium* comprises 8–10 species. These belong to the marine cyanobacteria and can form red to yellow blooms. (One freshwater species, *T. lacustre* Klebahn, is known). Janson *et al*. (1995) made a careful study of different species of *Trichodesmium* collected in 1991–94 from the Caribbean and Sargasso Seas and the Indian Ocean close to the coast of Zanzibar Island. They identified six species with the aid of both light and electron microscopy. However, only two species have been recorded as toxic.

Trichodesmium thiebautii has been shown to possess a neurotoxin similar in action to anatoxin-a. This planktonic tropical marine species has been shown to be toxic to some, but not all, marine invertebrates, which graze on it. Furthermore, there are reports of breathing difficulties from people who have been near red-tide blooms of *Trichodesmium*.

Colonies of *T. thiebautii* consist of many trichomes which are bundled together parallel or twisted in a rope-like fashion. A large fraction of the population may have trichomes in a radiate or spherical form (Fig. 19.7C). Colonies are usually buoyant and are about 1 mm × 3 mm in size. They usually appear golden-brown in colour, but can vary from grey to brown to red. Cell diameter ranges from 7 µm to 16 µm, and cells are usually as long as they are wide, or can be up to twice as long as wide. In September 1992, *T. thiebautii* was common in the Caribbean Sea (Janson *et al*., 1995) while other *Trichodesmium* species were rare.

Trichodesmium erythraeum has straight trichomes oriented parallel in bundles; cell width is 7–11 µm (rarely 21 µm) and cell length 5.4–11 µm. Most cells are shorter than they are wide and red in colour (Figs. 19.7A, B). *T. erythraeum* appears in blooms together with other *Trichodesmium* species. In December 1993, it was most common in the Indian Ocean close to the coast of Zanzibar (Janson *et al*., 1995).

19.4.1.3 *Nostocales*

***Anabaena* Bory ex Bornet et Flahault 1886 and *Anabaenopsis* (Woloszynska) Miller 1923**

Anabaena is a freshwater genus that is sometimes present in brackish and marine waters. This genus comprises more than 80 species distributed in the temperate zone as well in the tropics. Many species are similar in appearance and difficult to separate from each other. Table 19.3 presents the characteristic features of common *Anabaena* species. Within this genus, nine species are associated with toxin production. These toxins are the potent neuromuscular blocking alkaloid anatoxin-a, several of the paralytic shellfish poisons (PSP), the anticholinesterase organic phosphate anatoxin-a(s) and the hepatotoxic cyclic peptide microcystins. The species associated with one or more of these toxins include *Anabaena bergii*, *A. circinalis*, *A. farciminiformis*, *A. flos-aquae*, *A. macrospora*, *A. mendotae*, *A. lemmermannii*, *A. planctonica*, *A. spiroides* and *A. variabilis*. In addition, in the related genus *Anabaenopsis*, the species *A. milleri* has been shown to be toxic.

TABLE 19.3 Characteristic features of *Anabaena* species

Straight trichomes	Vegetative cells	Heterocytes	Akinetes	Trichomes	Mucilage layer	Habitat	References
A. planctonica Brunnthaler 1903	Short, barrel–shaped to spherical W 9–15 L up to 10	Spherical Ø 9–16	Ellipsoidal to cylindrical, distant W 9–21 L 15–30–35	Solitary, straight, long	+ Diffluent	Eutrophic	Geitler (1932); Komárková-Legnerová and Eloranta (1992)
A. smithii (Komárek) Watanabe 1991	Short, barrel–shaped to spherical W 9–15 L 9–10	Spherical Ø 7–12	Spherical, sometimes slightly angular Ø (12)15–30	Solitary, straight	+ Diffluent	Highly eutrophic, water-blooms common	Watanabe (1991)
A. macrospora Klebahn 1895*	Spherical to lemon–shaped Ø 5–6.5 or [5–7 × 6–9]	Spherical Ø 5–7	Single, seldom in pairs, separated, widely ellipsoidal, epispore burst at excentric line, polyhedral form 17–19 × 20–26	Solitary, Straight, sometimes bent	–	Eutrophic, Water–blooms	Komárková-Legnerová and Eloranta (1992)
A. viguieri Denis et Frémy 1923	Spherical or slightly barrel–shaped W 5.5–7.5 L 4–6	shorter than wide W 5–7 L 4–5.5	Widely ellipsoidal W 9–11 L 15–24	Straight or slightly curved	–	Eutrophic, not in masses. common	Komárková-Legnerová and Eloranta (1992)
A. bergii Ostenfeld 1908*	Spherical W 8 L <8	Spherical Ø 10	Ellipsoidal distant from heterocytes, brownish W 20 L 24	Solitary, tapering to ends, end cells conical, bluntly pointed	–	Eutrophic, higher conductivity. brackish water	Ostenfeld (1908); Couté and Preisig (1978)

…/…

TABLE 19.3 *(Suite)*

Straight trichomes	**Vegetative cells**	**Heterocytes**	**Akinetes**	**Trichomes**	**Mucilage layer**	**Habitat**	**References**
Coiled trichomes	Vegetative cells	Heterocytes	Akinetes	Trichomes	Mucilage layer	Habitat	References
A. crassa (Lemmermann) Komárková-Legnerová et Cronberg 1992	Spherical to barrel-shaped, slightly shorter than wide Ø 11–12.5–15	Spherical Ø 10–17	Widely ellipsoidal, distant from heterocytes W 13–22–25 L 15–28–35	Regularly spirally twisted	+ Dense, wide	Eutrophic	Komárková-Legnerová and Cronberg (1992)
A. circinalis Rabenhorst ex Bornet et Flahault 1888*	Spherical to barrel-shaped Ø 8–11–14	Spherical to widely ellipsoidal Ø 9–12	Elongated, ellipsoidal to cylindrical W 15–21 L 20–28	Wide regular spirals	– (+) Narrow, diffluent	Eutrophic	Komárek (1958)
A. spiroides Klebahn 1938*	Spherical to widely ellipsoidal Ø 7–8–8.6	Spherical Ø 7–9	Widely to elongated ellipsoid, slightly curved, distant from heterocytes W 10–14–15 L 17–22	Solitary, regularly or irregularly coiled	–	Water-blooms common	Komárková-Legnerová and Eloranta (1992)
A. compacta (Nygaard) Hickel 1985	Spherical Ø 4–5	Spherical Ø 3–5	Widely ellipsoidal W 6–7 L 7.7–10	Solitary, regularly coiled 200–300 µm 20–30 spirals	+ Narrow	Eutrophic	Hickel (1985)
A. flos-aquae (Lyngbye) Brébisson ex Bornet et Flahault 1888*	Spherical to slightly ellipsoidal Ø 4–5	Spherical to slightly ellipsoidal Ø 4–5	Ellipsoidal to cylindrical, slightly arcuate, apart from heterocytes, one to three in a row W 5.5–13 L (13)–15–35	Irregularly, rarely regularly twisted forming clusters	_	Eutrophic	Starmach (1966); Komárková-Legnerová and Eloranta (1992)

…/…

TABLE 19.3 *(Suite)*

Straight trichomes	Vegetative cells	Heterocytes	Akinetes	Trichomes	Mucilage layer	Habitat	References
A. lemmermannii var. *lemmermannii* P. Richter 1903*	Elongated cylindrical with rounded ends W 3–4 L 4–8–12	Shortly ellipsoidal or ovoid, disposed between two akinetes W 4–6 L 5–7	Elongated cylindrical with rounded ends, straight or slightly bent, next to heterocyte, star-like disposed W 6–7–10 , L 15–23–29	Irregularly loosely twisted, clusters	+ Weak, diffluent	Mesotrophic to oligotrophic	Komárkova (1988)
A. lemmermannii var *minor* (Utermöhl) Komárková-Legnerová 1988	Spherical to shortly barrel-shaped Ø 3–4.5–(9)	Spherical to widely disposed between two akinetes, Ø 4–6	Elongated cylindrical with rounded ends, straight or slightly bent, next to heterocyte, star-like disposed W 6–9, L 11–22	Individual or loosely irregularly twisted	+ Weak, diffluent	Eutrophic lakes, humic character, frequent in Europe	Komárkova (1988)
A. mendotae Trelease 1889*	Cylindrical, longer than wide W 2.5–4 L 6–10	Widely ellipsoidal W 4–7 L 5.4–7–11	Long cylindrical, slightly bent W 5.5–7 L 16–30–40	Irregularly twisted in loops, individual large circles	–	Slightly eutrophic lakes	Komárková-Legnerová and Eloranta (1992)
A. perturbata var. *tumida* (Nygaard) Cronberg et Komárek 1992	Spherical Ø 6–7.5–9	Spherical Ø 5.5–6	Wide kidney-shaped W 10–13.5 L 18–23	Coiled circular	–	Eutrophic, scarce	Cronberg and Komárek (1992)
A. farciminiformis Cronberg et Komárková-Legnerová 1988*	Kidney-shaped W 4.3–4.8–5.7 L 5.9–17.1	Spherical to ellipsoidal W 6.4–8.6 L 7.1–10	Cylindrical, arcuate, rounded ends W 7.1–8.6 L 22.2–30	Twisted irregular spirals	–	Eutrophic	Cronberg and Komárková (1988); Willén and Mattson (1997)

* Toxic or harmful species; ø: diameter; W: width; L: length; dimensions in micrometres.

Both *Anabaena* and *Anabaenopsis* genera are filamentous cyanobacteria with more or less spherical cells. The trichomes resemble string of beads. The trichomes may be embedded in mucilaginous matrix and sometimes form large colonies. They have differently shaped heterocytes (nitrogen-fixing cells) and akinetes (spores or resting cells). The position of the heterocytes on the filaments is an important diagnostic feature. *Anabaena* has single heterocytes intercalary (mid-trichome) positioned on the filament. *Anabaenopsis*, on the other hand, has terminal and paired, intercalary heterocytes.

Anabaena circinalis has widely coiled trichomes, regularly or irregularly twisted, spirals 70–120 µm wide, free-floating or in larger colonies (Fig. 19.4G). Cells are spherical to short, barrel-shaped with many aerotopes, 7–11 µm in diameter and with heterocytes 7–13 µm wide. Akinetes are 25–30 µm long and 12–13 µm wide.

Anabaena farciminiformis is planktonic with irregularly coiled trichomes of different lengths. Cells are short, sausage-shaped, pale blue-green in colour. Heterocytes are spherical or slightly elongated. Cell dimensions are 5–17 µm × 4–8 µm. Akinetes are long cylindrical, 7–9 µm wide and 23–30 µm long and situated a distance from the heterocytes (Fig. 19.4A). *A. farciminiformis* was found as a monoculture in a small eutrophic pond in South Scania, Sweden (Cronberg and Komárková, 1988). It was shown to be hepatotoxic by the mouse bioassay (Willén and Mattsson, 1997).

Anabaena flos-aquae is planktonic and has irregularly coiled trichomes that may be either solitary or in a twisted mass (Fig. 19.4L). Cells are spherical to barrel-shaped, 2.5–7 µm wide and 2.5–8 µm long. Heterocytes are spherical to elliptical, 5–9 µm in diameter and a distance from the akinetes. Cell contents have conspicuous aerotopes, and cells link up in bead-like fashion to form trichomes. Akinetes are cylindrical or sausage-shaped, solitary or in pairs, 13–35 µm long and 5.5–13 µm wide and a distance from the heterocytes.

Anabaena lemmermannii is similar to *A. flos-aquae*, but differs in that the akinetes are adjacent to, rather than a distance from, the heterocytes. There exist many different forms of *A. lemmermannii* (Table 19.3), which thrives in different types of water body. *A. lemmermannii* forms very characteristic colonies (Fig. 19.4H). Heterocytes and akinetes gather in the middle of the colony with the coiled filaments of vegetative cells outside, giving the colony a flower-like appearance (Figs. 19.4H, I). This species in different forms is common in water-blooms in eutrophic lakes of the temperate region.

Anabaena macrospora has straight filaments embedded in wide mucilage. They are free-floating and solitary. Cells are spherical to barrel-shaped, sometimes longer than they are wide, dimensions 5–9 µm × 5–6.5 µm. Heterocytes are spherical, 5–7 µm in diameter. Akinetes are thick-walled, short cylindrical, slightly hexagonal, 17–19 µm wide and up to 26 µm long (Fig. 19.4D). The akinetes burst in a characteristic way at one end. *A. macrospora* is common in eutrophic lakes. The species probably has a cosmopolitan distribution.

Anabaena mendotae has irregular, loosely twisted, narrow filaments without mucilage (Fig. 19.4B). Cells are cylindrical to barrel-shaped, 5.5–10 µm long and 5–6 µm wide with many aerotopes. Heterocytes are spherical, 5–6 µm in diameter, and akinetes are cylindrical, 16–30 µm long and 5–11 µm wide, slightly bent, and a distance from the heterocytes. *A. mendotae* appears in mesotrophic lakes, often together with other cyanobacteria.

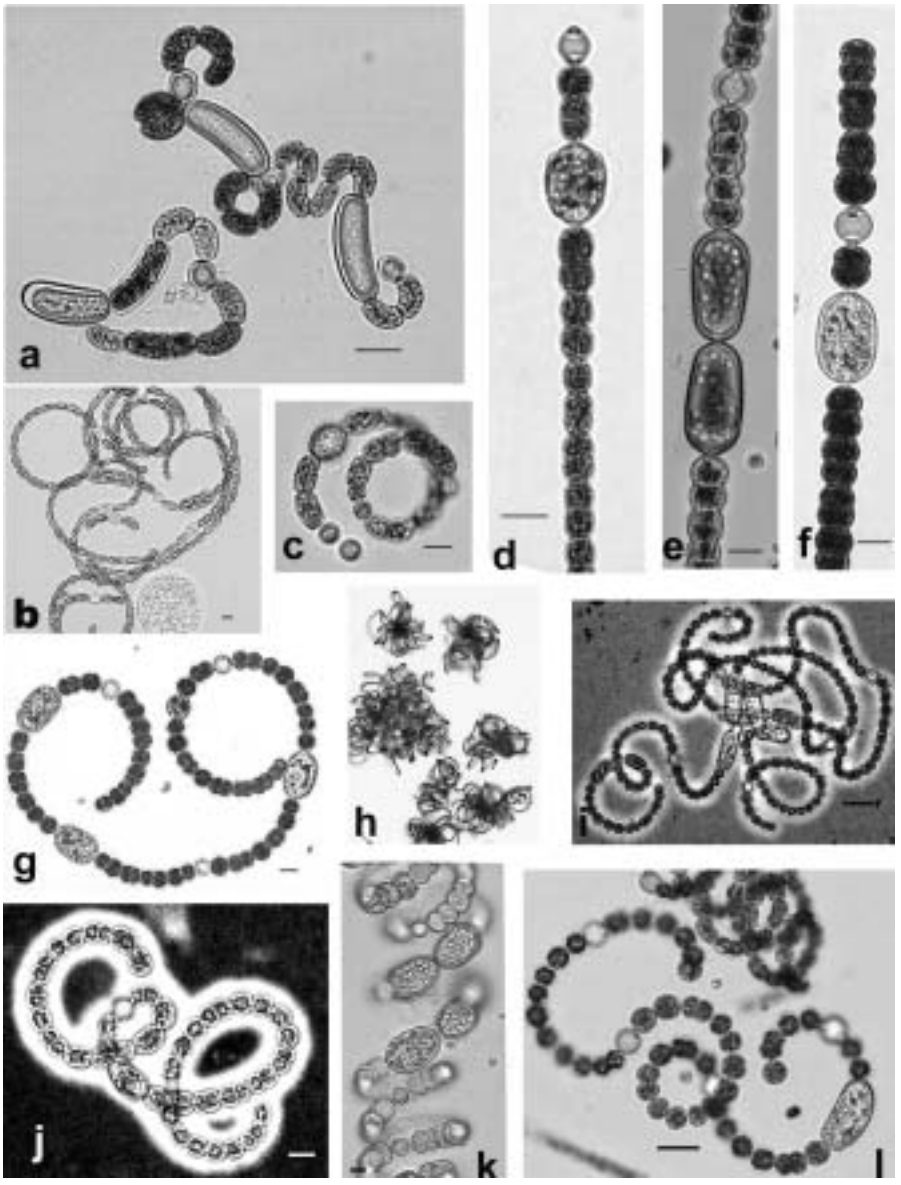

Figure 19.4
A, *Anabaena farciminiformis*; B, *A. mendotae*; C, *Anabaenopsis milleri*; D, *Anabaena macrospora*; E, F, *A. planctonica*; G, *A. circinalis*; H, I, *A. lemmermannii*, J. *A. spiroides*; K, *A. crassa*; L, *A. flos-aquae*. Scale bar = 10 µm.

Anabaena planctonica has solitary, straight filaments embedded in a mucilaginous sheath up to 30 µm wide (Figs. 19.4E, F). The vegetative cells have aerotopes and are spherical to barrel-shaped, often shorter than wide, with dimensions 9–15 µm wide and up to 10 µm long. Heterocytes are spherical and are more or less the same width as the vegetative cells. Akinetes are ellipsoidal, sometimes in pairs and a distance from the heterocytes, 10–20 µm wide and 15–30 µm long. *A. planctonica* is common in water-blooms of lakes and ponds in the temperate zone.

Anabaena spiroides has regularly twisted trichomes, which are enclosed in a mucilaginous sheath (Fig. 19.4J). Cells are spherical or compressed-spherical, 6–8 µm in diameter, and the heterocytes are similar in size and form to the vegetative cells. Akinetes are initially nearly spherical, but later wide oval to cylindrical, with dimensions 17–21 µm × 10–17 µm. They are rounded with slightly asymmetric ends. *A. spiroides* might be difficult to separate from *A. flos-aquae*; however, *A. spiroides* has larger cells and the trichomes are embedded in a mucilaginous matrix.

Anabaena variabilis trichomes have cells, which are compressed globose, 3.7–6.5 µm in diameter, and heterocytes are globular or ovate 5.5–8 µm in diameter and 5.8–6.5 µm long. Akinetes are ovate, from 6.8–9 µm wide and 7.5–14 µm long. This alga appears in benthos and plankton and in fresh and brackish water-bodies.

Anabaenopsis (Woloszynska) Miller 1923

Species in the genus *Anabaenopsis* have terminal and paired intercalary (mid-trichome) heterocytes. The genus comprises about 20 species with more species in the tropics than in the temperate zone. They thrive in water with higher conductivity and are common in brackish water.

Anabaenopsis milleri has spiral trichomes, which have from 2.5, or more, complete spirals with cylindrical to barrel-shaped cells from 6–7 µm wide and 8 µm long. The terminal heterocytes are spherical and 5–8 µm in diameter. Akinetes are spherical, c. 11 µm in diameter, single or in pairs at a distance from the heterocytes (Fig. 19.4C). *A. milleri* appears in brackish waters.

Aphanizomenon Morren ex Bornet et Flahault 1886

The genus *Aphanizomenon* comprises about 20 species. It is most common in the temperate zone, where several species appears in dense water-blooms. *Aphanizomenon* is rare in the tropics; however, some species have only a tropical distribution. Sometimes it is difficult to separate Anabaena species with straight trichomes from *Aphanizomenon*. However, Anabaena species have distinct constrictions at the cross walls; most *Aphanizomenon* species have no constrictions. *Aphanizomenon* have straight trichomes, rarely twisted. They are mostly planktonic, appearing as solitary trichomes or as aggregations of filaments (flakes), which can reach a size of 10–15 mm. *Aphanizomenon* most often gather at the surface, but can make vertical migrations. The trichomes can regulate their position in the water column with the help of aerotopes.

In the most-used taxonomic literature (Smith, 1920; Geitler; 1932; Huber-Pestalozzi; 1938; Komárek, 1958; Desikachary, 1959; Prescott, 1962) only one to four *Aphanizomenon* species are described. Thus when identifying *Aphanizomenon* with the above-mentioned literature, researchers try to find the description with the best fit. However, the alga might be slightly different in size, morphology, habitat and distribution. Therefore we have included the most common *Aphanizomenon*

species in Table 19.4, although they have not been reported as toxic. More *Aphanizomenon* species might have been recorded as toxic, but have been given the collective name *A. flos-aquae* or just *Aphanizomenon* sp.

Aphanizomenon flos-aquae produces the paralytic shellfish poisons saxitoxin and neosaxitoxin. The species is present in fresh and brackish water environments. It has been associated with toxic events in Canada, the United States and Europe. Colonies are usually very buoyant and present near the water surface. Its trichomes are typically arranged in parallel as a colony, or as individual trichomes. When preservative is added, trichomes in colonies often disperse. Cells are 5–8 µm in diameter and longer than they are wide, becoming progressively longer close to the ends of the trichomes (Figs. 19.6A–C). Cells are cylindrical, giving a straight, almost flat-sided appearance to the trichomes. Heterocytes are 5–9 µm wide and 10–18 µm long. Akinetes are intercalary, at a distance from the heterocytes, 6–11 µm wide and 40–220 µm long.

Aphanizomenon flos-aquae var. *klebahnii* is similar to *A. flos-aqaue*, but in var. *klebahnii* all cell dimensions are smaller (Table 19.4), also the flakes are less buoyant than in *A. flos-aquae* (Figs. 19.6D–F). In recent literature var. *klebahnii* has been raised to species level, although no valid separation of the two varieties yet has been published.

In 1991 the species *Aphanizomenon yezoense* was described from Japan by Watanabe. It was found in a mesotrophic dam constructed in the Hokkaido District. Now it has been recorded from Greece and Sweden, where it is a common member of toxic water-blooms. The trichomes are gathered in bundles or flakes, but these are much smaller than those in *A. flos-aquae* or *A. flos-aquae* var. *klebahnii* (Figs. 19.6G–I).

Aphanizomenon gracile appears in the plankton as solitary, straight trichomes. It is smaller than *A. flos-aquae* in all dimensions. Cells are cylindrical, 2–3 µm wide and 2–6 µm long. Heterocytes are more or less the same size and form as vegetative cells. Akinetes are cylindrical with dimensions 22–30 µm × 4.5–5.5 µm. *A. gracile* is frequent in slightly eutrophic water-bodies. Pereira *et al*. (2001) found that it produced PSP toxins.

Aphanizomenon ovalisporum has free-floating, solitary, straight or slightly bent trichomes, 0.5–1.5 mm long. Cells are cylindrical, 3–5 µm wide and 6–7 µm long. Heterocytes are spherical to oval, 5–7 µm wide and 8–12 µm long. Akinetes are oval with dimensions 18–20 µm × 12–14 µm (Fig. 19.5F). *A. ovalisporum* was first described from Italy (Forti, 1911) and later from Lake Kinneret, Israel (Gophen, 1994). *A. ovalisporum* was isolated from Lake Kinneret and showed the presence of cylindrospermopsin. This was the first report on the toxicity of this alga. Recently it was also found to be toxic in Australia.

Cylindrospermum Kützing ex Bornet et Flahault 1886

The genus *Cylindrospermum* is characterized by uniformly short trichomes without sheath, but often in common mucilage. The cells are cylindrical and have constrictions at the cross walls. *Cylindrospermum* has terminal heterocytes, in both ends or only at one end, sometimes intercalary. Akinetes are adjacent to the terminal heterocytes, spherical-oval-cylindrical in form and much larger than the vegetative cells (Fig. 19.8C). *Cylindrospermum* has no aerotopes, thus it is most often found in the benthos, and very common in the tropics. In Finland, *Cylindrospermum* sp. was isolated and found producing neurotoxins (Sivonen *et al*., 1989).

TABLE 19.4 Characteristic features of *Aphanizomenon* species

Species	Vegetative cells	Terminal cells	Heterocytes	Akinetes	Trichomes	Habitat	References
A. flos-aquae* Ralfs ex Bornet et Flahault 1886							
Diagnostic features	Cylindrical	Hyaline, cylindrical, rounded ends	Cylindrical	Elongated cylindrical	Form fascicles, cell width the same over the whole trichome, constrictions at the cross walls	Polluted and eutrophic	Komárek and Kovácik (1989)
Diameter	(4.6) 5–6.8 (7.8)	(4) 4.3–5 (6.4)	5–6.4	7.1–10		water also brackish water	
Length	(5) 5.7–12.1 (13.6)	(7.1)10.7–18.6 (24.3)	10–14.3 (24.3)	(42.8) 54.3–82.8 (88.5)	bundles 80–150–2000.		
A. var. klebahnii* Elenkin 1909							
Diagnostic features	Cylindrical	Hyaline, cylindrical, rounded ends	Elliptic	Elongated cylindrical	Form spindle-like bundles	Eutrophic water bodies	Komárek and Kovácik (1989)
Diameter	4.3–5.5 (5.7)	2.8–4.5 (5)	3.1–5 (5.7)	(5.5) 7.1–8.6 (9.3)		also brackish water	
Length	(4.3) 5.7–10.7 (11.4)	(7.1) 8.6–15.7 (17.1)	(6.4) 7.1–10.7 (11.4)	(30) 35.7–47.1 (54.3)	bundles up to 500		
A. gracile* (Lemmermann) Lemmermann 1907							
Diagnostic features	Barrel-shaped	Narrowing, not hyaline	Round to cylindrical	Cylindrical with cup-like structures at the ends	Solitary	Eutrophic, also brackish	Watanabe (1991)
Diameter	(2.3) 2.6–3.1 (3.7)	1.7–2.1 (2.8)	3.4–4.9 (5.9)	(2.9) 3.9–5.9 (6.4)		water	
Length	(2.6) 2.8–6.4 (7.1)	(3.6) 4.3–6.4 (7.4)	3.9–7.8 (9.8)	(6.4) 7.8–14.7(16.7)	20–30–60		

…/…

TABLE 19.4 *(Suite)*

Species	Vegetative cells	Terminal cells	Heterocytes	Akinetes	Trichomes	Habitat	References
***A. skujae* Komárková-Legnerová and Cronberg 1992**							
Diagnostic features	Cylindrical	Narrow, elongated with ends bluntly pointed	Oval to cylindrical	Cylindrical with rounded ends	Solitary	Eutrophic	Komárková-Legnerová and Cronberg
Diameter	1.7–2.1–2.6		1.9–2.8–3	2.7–3.6–3.7			(1992)
Length	4.2–6.6–16.8		7.5–8.5–11.4	25–27–34 (40)	Up to 300		
***A. yezoense* Watanabe 1991**							
Diagnostic features	Cylindrical	Elongated, cylindrical, with abruptly rounded ends	Cylindrical	Long, cylindrical	Solitary or in bundles, not constricted at cross walls	Mesotrophic	Watanabe (1991)
Diameter	2.7–4.0	2.8–4.0	3.8–5.1	4.7–7.3			
Length	3.1–7.8	10.9–28.8	5.4–11.4	31.2–48.9	100–180–330		
***A. issatschenkoi* (Usacev) Proskina-Lavrenko**							
Diagnostic features	Oval	Pointed end cells	Oval to cylindrical	Oval to cylindrical	Solitary	Eutrophic water bodies	Watanabe (1991)
Diameter	2.6–3.5–4.4	1.2–1.9–2.9	3.3–4.1–6.2	4.3–5.6–7.0			
Length	5.5–8.8–15.9	8.7–22.6–34.8	7.0–8.8–10.9	9.3–14.1–18.7	180–600		
A. ovalisporum* Forti							
Diagnostic features	Cylindrical	Rounded	Oval	Oval to barrel-shaped	Solitary, constricted at cross walls	Mesotrophic	Forti (1911)
Diameter	4–5	3–5	5–7	10–12–14			Banker *et al*. (1997)
Length	6–7	25–30	8–12	8–14–20	500–1500		

* Toxic species; dimensions in micrometres.

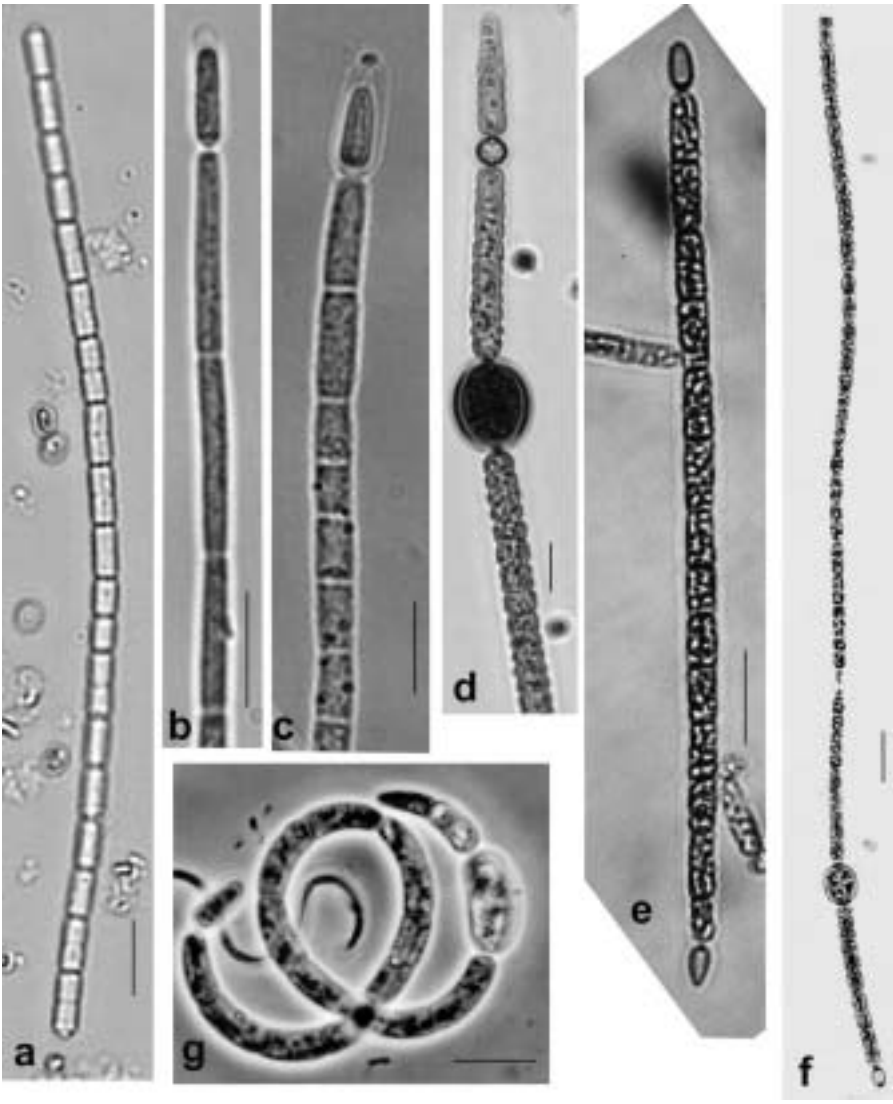

Figure 19.5
A, *Pseudanabaena* cf *catenata*; B, *Cylindrospermopsis africana* from Lake Victoria, East Africa; C, C. *raciborskii* from Lake Kariba, Zimbabwe; D, *Anabaena bergii* from Sri Lanka; E, *C. raciborskii*; F, *Aphanizomenon ovalisporum*; G, *C. raciborskii*, curved. Scale bar = 10 µm.
Sources: F, G, Peter Baker and Larelle Fabbro.

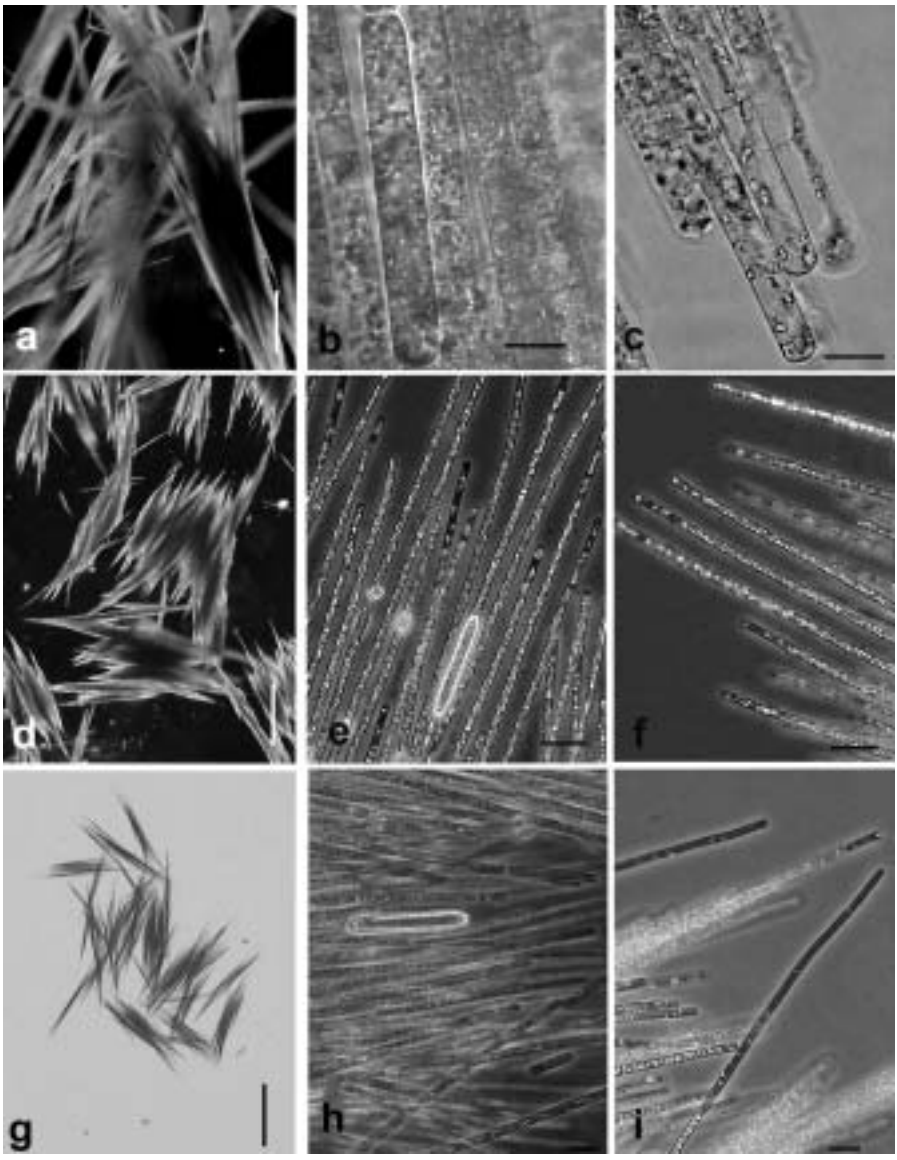

Figure 19.6
A–C, *Aphanizomenon flos-aquae*; D–F, *A. flos-aquae* var. *klebahnii*; G–I, *A. yezoense*. Scale bar = 100 µm (A, D, G); 10 µm (others).

***Cylindrospermopsis* Seenayya et Subba Raju 1972**

The genus *Cylindrospermopsis* now contains eight species, which are distributed throughout the tropical and subtropical zones (Komárek and Kling, 1991; Komárková-Legnerová and Tavera, 1996; Padizak, 1997). The most common species is *C. raciborskii*, which has also been shown to be toxic and produce the toxin cylindrospermopsin (Hawkins *et al*., 1985; McGregor and Fabbro, 2000; Schembri st al. 2001).

The first record of *Cylindrospermopsis raciborskii* was from Java and at that time it was named *Anabaena raciborskii* (Woloszynska, 1912). Later it was transferred into the genus *Anabaenopsis* because it had terminal heterocytes, and became *Anabaenopsis raciborskii* (Miller, 1923). However, as the formation of heterocytes was not done in the same way in *A. raciborskii* as in the other *Anabaenopsis* species, Seenayya and Subba Raju (1972) created the new genus *Cylindrospermopsis* and transferred *Anabaenopsis raciborskii* into this genus as *Cylindrospermopsis raciborskii* (Horecká and Komárek, 1979).

Cylindrospermopsis raciborskii has solitary, straight or slightly curved filaments with a length up to 200 µm. Cells are cylindrical, slightly constricted at the cross walls, 2.5–4 µm wide and 2.5–16 µm long. The terminal heterocytes are long and conical with dimensions 5–6 µm × 2–2.5 µm. Akinetes are elongated oval and situated adjacent to the heterocyte or terminal vegetative cell, 2.8–3.2 µm wide and 4.5–11(16) µm long (Figs. 19.5C, E, G). The related species *C. africana* Komárek and Kling differs from *C. raciborskii* by having longer and thinner cells (Fig. 19.5B). *C. raciborskii* appears in eutrophic ponds, lakes, dams and rivers.

***Gloeotrichia* J. Agardh ex Bornet et Flahault 1886**

Gloeotrichia has round, macroscopic colonies, 1–3(8) mm in diameter with radially arranged trichomes, which are provided with a sheath at the base. The trichomes have a terminal heterocyte at the basal end and terminate in a hair-like filament at the other end. The vegetative cells are cylindrical; the whole colony is embedded in mucilage. Akinetes are formed adjacent to the terminal heterocytes (Figs. 19.8A, B). The genus *Gloeotrichia* comprises 14–20 species (Geitler, 1932; Desikachary, 1959).

Gloeotrichia echinulata is found in mesotrophic lakes and can form waterblooms. The basal vegetative cells are 5.5–10 µm wide and at the opposite end hair-like cells are only 2 µm wide (Figs. 19.8A, B). As early as 1954 it was described as hepatotoxic by Ingram and Prescott (1954).

***Hormothamnion* Grunow 1867**

The genus *Hormothamnion* is comprised of two species, which grow in macroscopic, bush-like colonies in marine environments. Heterocytes are intercalary but akinetes have never been recorded.

Hormothamnion enteromorphoides is widely distributed in tropical regions and has toxic strains. The species has hepatotoxic effects (Gerwick *et al*., 1986). The colonies are bush-like with a thin sheath up to c. 2 cm long and grow along the coast on the sediment surface. Trichomes are 7–9 µm wide. Cells are barrel-shaped with intercalary heterocytes, 6–7 µm wide and 7–10 µm long.

***Nodularia* Mertens ex Bornet et Flahault 1886 nom. cons.**

The genus *Nodularia* comprises seven revised species (Smarda *et al*., 1988; Komárek *et al*., 1993), the best known of which is *Nodularia spumigena*, which also

has a worldwide distribution. *N. spumigena* is well known for its toxic blooms, which occur in the Baltic Sea and brackish lakes and estuaries of Australia, New Zealand, the United States and Uruguay. It can live either in the plankton or attached to macroalgae or sediments. The toxin produced is the cyclic pentapeptide nodularin. The filamentous colonies of *N. spumigena* have trichomes enclosed with more or less firm sheaths (Figs. 19.7D, E). The unbranched filaments have disc-shaped vegetative cells as well as disc-shaped heterocytes. Filaments are straight or often twisted, and cells are constricted at cross walls, from 8–14 µm in diameter and only about 3–5 µm long. The sheath is thin and colourless. Heterocytes are compressed and elliptical in shape, 14 µm in diameter and 7 µm long. Akinetes are transversely oval, 6–11 µm × 10–12 µm and rarely spherical, 8–10 µm in diameter, with brownish cell wall, in series, rarely solitary or in pairs.

***Nostoc* Vaucher ex Bornet et Flahault 1886**

The genus *Nostoc* comprises more than 40 species, according to Geitler (1932). They are terrestrial or waterborne with mostly macroscopic, mucilaginous colonies of different appearance, free-floating or attached to substrates. They have twisted trichomes embedded in slime. *Nostoc* was previously reported as a toxigenic genus with *N. rivulare*, which produced microcystins (Davidson, 1959). Also *N. linckia*, *N. paludosum*, *N. zetterstedtii* and *Nostoc* sp. have been found producing microcystins (Table 19.1). In Finland the strain *Nostoc* nr 152 was isolated and was found to produce about nine hepatotoxic peptides similar to those reported from other cyanobacteria (Sivonen, 1990; Sivonen *et al*., 1990).

Nostoc linckia has macroscopic round colonies, at first attached, later free-floating, irregularly formed, with diffluent mucilage, blue-green, light violet, dirty-green to brown in colour. Trichomes are twisted, cells short and barrel-shaped, 3.5–4 µm wide, heterocytes spherical 5–6 µm in diameter and akinetes are mostly spherical, 6–7 µm wide and 7–8 µm long (Fig. 19.8D).*N. linckia* appears in stagnant, fresh or marine waters, can form large masses and is cosmopolitan in distribution.

Nostoc paludosum produces small, round, slimy, free-floating colonies hardly visible to the naked eye. Trichomes are loosely intertwined with wide colourless or yellowish sheaths. Cells are pale blue-green, barrel-shaped, 3–3.5 µm wide, and have more or less the same length as width. Heterocytes are slightly larger than the vegetative cells. Akinetes are oval, 4–4.5 µm wide. *N. paludosum* is cosmopolitan and appears in stagnant waters.

Nostoc rivulare colonies are at first spherical, later irregularly dissolved. The sheath surrounding the trichomes is yellowish only in the periphery of the colony. Cells are almost spherical, 5–6 µm in diameter. Heterocytes are ellipsoidal, 5 µm wide and 6–10 µm long. Akinetes are elongated, 6–8 µm wide and 8–14 µm long, with a rough, pale-brown cell wall. The species, which grows on wet soil and cliffs, has a cosmopolitan distribution.

The juvenile *Nostoc zetterstedtii* consists of minute balls. The adult colonies are aggregated small spheres forming macroscopic, warty, cartilaginous balls, up to 10 cm in diameter. Inside the stiff balls the living trichomes are gathered at the surface while the centre of the ball is dead, or sometimes colonized by bacteria and/or fungi. Cells are spherical to barrel-shaped, 4 µm wide. Heterocytes are spherical, single or several together, 8–15 µm wide. Akinetes are absent. The balls of *N. zetterstedtii* are loose, typically lying on the bottom or on the submerged vegetation in oligotrophic, alpine and Scandinavian lakes (Mollenhauer *et al*., 1999).

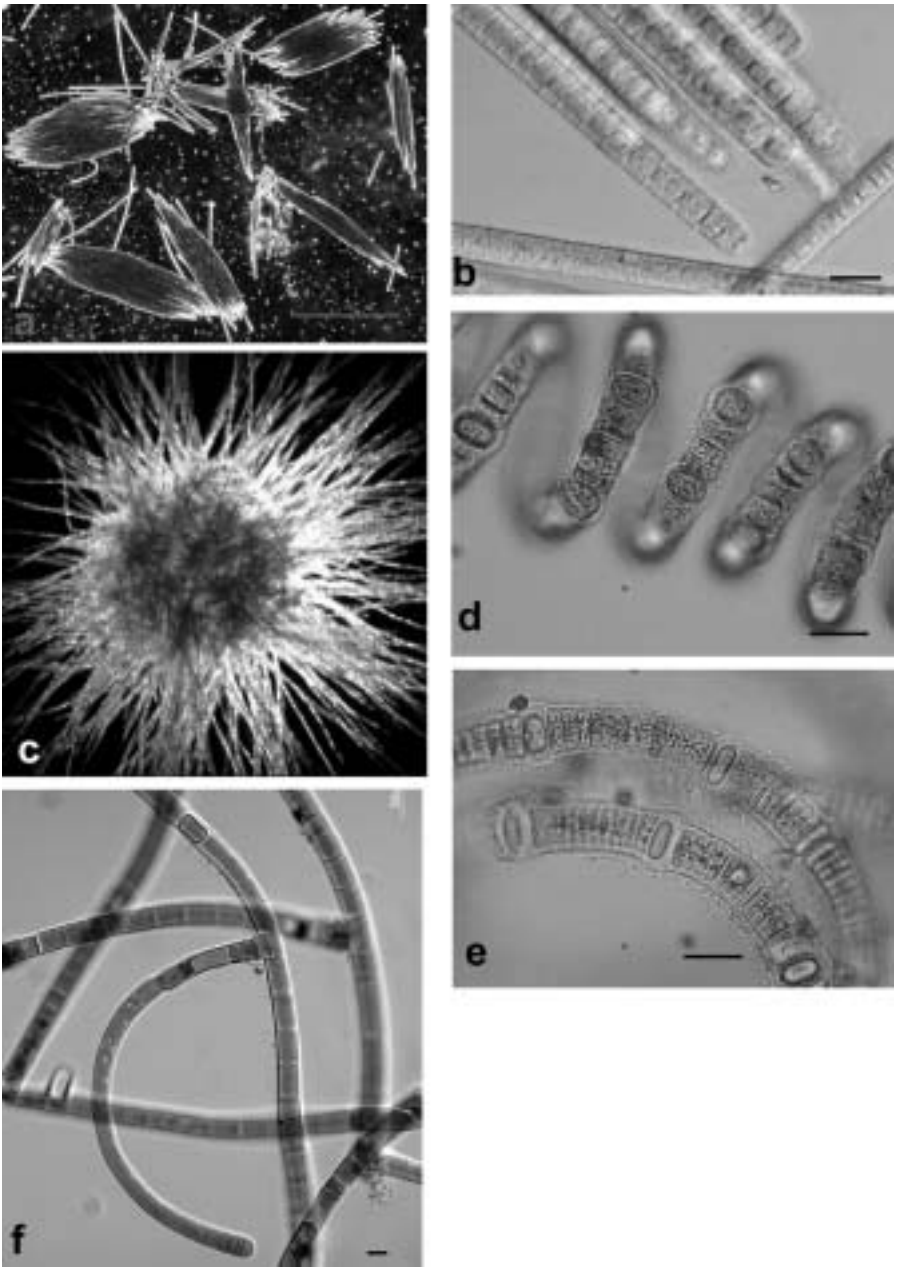

Figure 19.7
A, B, *Trichodesmium erythraeum*; C, *T. thiebautii*; D, E, *Nodularia spumigena*; F, *Hapalosiphon fontinalis*. Scale bar = 100 µm (A); 10 µm (B–F).
Sources: A, C, Hans Paerl; F, Roland Bengtsson.

19.4.1.4 *Stigonematales*

***Fischerella* Gomont 1895**

The genus *Fischerella* comprises about 10 species. They live aerophytically on tree trunks, and also in stagnant water on stones and sediment. Only freshwater species have been recorded.

Fischerella epiphytica forms brown tufted mats or cushions on submersed tree trunks in the tropics. The filaments have mostly two rows of cells and exhibit true branching. Branches arise by cell division in a direction perpendicular to the main filament axis. Filaments are 15–20 µm wide. The cells are spherical or depressed globose with a brown sheath. Branches are upright cylindrical, 11–14 µm wide, and heterocytes are rare. Hormogonia consist of two to six cells (Fig. 19.10D). *F. epiphytica* produces neuro- and hepatotoxins (Ransom *et al*., 1978).

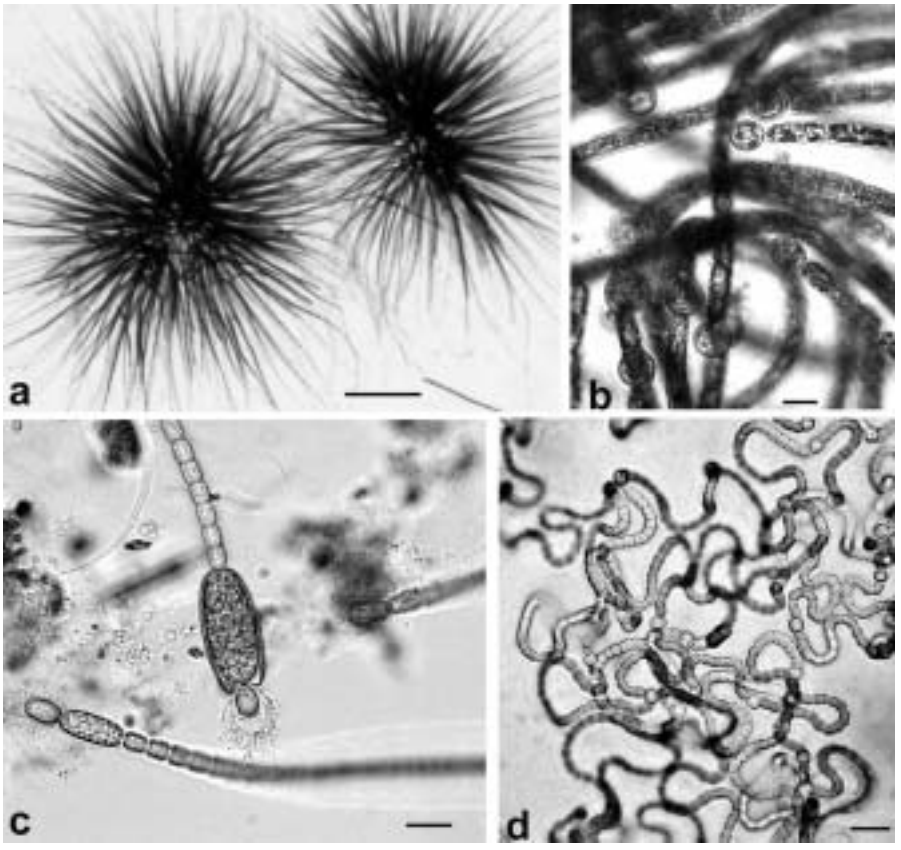

Figure 19.8
A, B, *Gloeotrichia echinulata*; C, *Cylindrospermum* sp., Australia; D, *Nostoc linckia*, Australia. Scale bar = 250 µm (A); 10 µm (B, D). *Sources:* D, Larelle Fabbro and Peter Baker.

***Hapalosiphon* Nägele in Kützing ex Bornet et Flahault 1886**

The thallus consists of twisted filaments, which are unilateral branched. The main filament has only one row of cells. The side branches have the same morphology as the main filament, but are thinner. Heterocytes are mostly intercalary and hormogonia are made from the side branches.

Hapalosiphon hibernicus and *H. fontinalis* have more or less the same filament structure with side branches, although they differ in size. The main branches of *H. hibernicus* are 7.2–9 µm wide with many side branches, 4.5–5 µm wide. Cells are rounded, quadrangular, and three to four times longer than they are wide. Heterocytes are cylindrical and 5 µm wide. *H. fontinalis* is much larger than *H. hibernicus* in all dimensions. *H. fontinalis* makes blue-green to brown, macroscopic mats, 1–3 mm high. The main branches are creeping, twisted, 12–24 µm wide with many side branches, 9–12 µm wide (Fig. 19.7F). The cells on the side branches are mainly cylindrical, one to two times longer than they are wide. Both species grow in stagnant water and have a cosmopolitan distribution.

***Schizothrix* Kützing 1843**

Schizothrix calcicola is a filamentous species that characteristically has numerous trichomes enclosed in a mucilaginous sheath (Fig. 19.9F). The sheath can be rather wide and laminated. Cells are 1–2 µm wide and 2–6 µm long. Trichomes within the sheath typically twist around each other. It is often found growing attached to a substrate in freshwater and marine locations, and has been implicated in toxic events near certain Pacific Ocean islands. The toxin produced is debromoaplysiatoxin, and exposure to it causes a 'swimmer's itch'. Furthermore, the toxin can be accumulated by the sea hare (*Stylocheilus longicauda*).

***Scytonema* Agardh 1824**

The genus *Scytonema* has free filaments, often in bundles and twisted in different ways. These creep along the substrate or are upright with false branching and thick, layered sheaths. Heterocytes are mainly intercalary and homogonia are produced at the end of the trichome, however akinetes are missing (Geitler, 1932).

Scytonema mirabile and *S. ocellatum* have similar filament morphology, but different macroscopic colonies. *S. ocellatum* grows in cushion-like layers, and is black or grey-blue in colour. The filaments are twisted together, 10–19 µm wide and up to 3 mm in length. with a brownish, layered, thick sheath. Cells are 6–14 µm wide, olive-green, quadrangular or little shorter than their width. Heterocytes are quadrangular to cylindrical (Fig. 19.10C). *S. ocellatum* grows on cliffs, wet soil and walls. *S. mirabile* has longer filaments than *S. ocellatum*, up to 12 mm, and a wider yellowish sheath (Fig. 19.10B). The filaments can aggregate and form macroscopic balls, black-brown or black-green, rarely blue-green, in colour.

***Tolypothrix* Kützing 1845**

Filaments have a firm sheath with one trichome per sheath. *Tolypothrix* has false branching, which appears below the heterocyte. Akinetes are missing. It grows aerophytically, on cliffs, wet soil and in water. *Tolypothrix byssoidea* has irregular false branching. The filaments can be up to 1 mm long and 10–15 µm wide. The branches are short, bent with a thin sheath, and yellow to brown in colour. Cells are 9–11 µm wide, barrel-shaped and shorter than they are wide (Fig. 19.10A).

***Umezakia* Watanabe 1987**

An aberrant-looking alga was found in plankton collected from Lake Mikata, Japan. It was isolated and cultivated under different conditions (Watanabe, 1987). At first sight this alga looked similar to *Raphidiopsis mediterranea* Skuja, but closer examination showed that it also had heterocytes and true branching. Obviously it was a new member of the order Stigonematales and was given the generic name *Umezakia*. The filaments consist of a single row of cells, sometimes with true

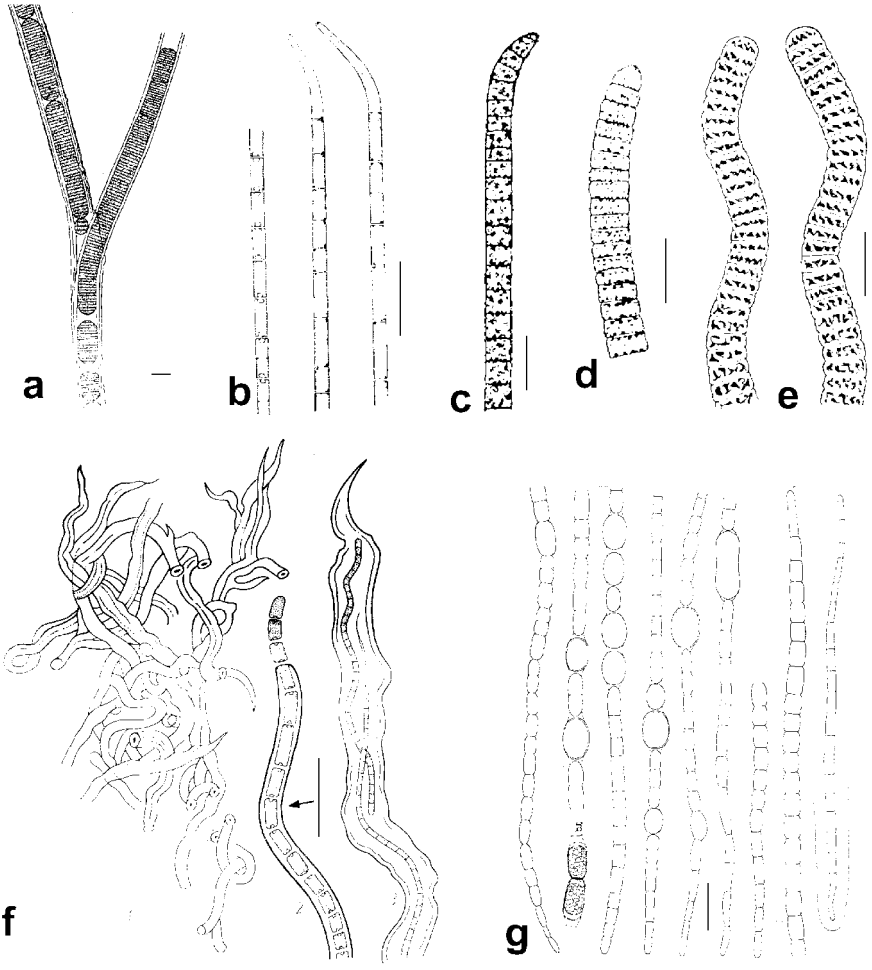

Figure 19.9
A, *Lyngbya wollei*; B, *Oscillatoria acutissima*; C, *Phormidium formosum*; D, *Oscillatoria nigro-viridis*; E, *Planktothrix planctonica*; F, *Schizothrix calcicola*; G, *Umezakia natans*. Scale bar = 10 µm.
Sources: A–D, Geitler (1932); E, F, Kondrateva (1968); G, Watanabe (1987).

branching of different kinds. The branches are initiated from longitudinal division of the parental cells. Sheaths are hyaline and watery. It has intercalary heterocytes and akinetes. The isolate was later tested for algal toxins and Harada *et al*. (1994) found that it produced cylindrospermopsin.

The species *Umezakia natans* has solitary, free-floating, straight or slightly curved trichomes with a thick mucilaginous sheath and sometimes with true branching. Trichomes attenuated at the ends, slightly constricted at the cross walls, were 50–3,000 µm long and 5–6 µm wide. At the apical part the cells are 2.5–3.5 µm wide. Cells are cylindrical to barrel-shaped, one to two times as long as they are wide. Heterocytes are spherical to elongate, 6–8 µm wide and 7–9 µm long. Akinetes appear single or in series, ellipsoidal, thick-walled with a smooth surface, 7–11 µm wide and 11–19 µm long (Fig. 19.9G). To date this species has only been recorded from Japan.

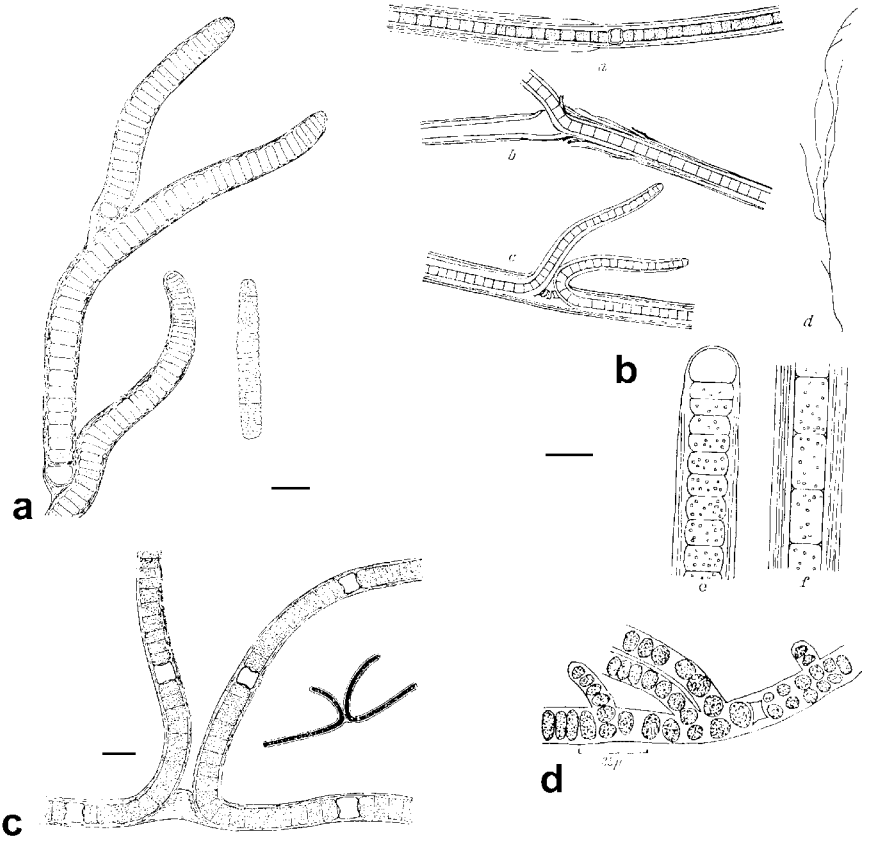

Figure 19.10
A, *Tolypothrix byssoidea*; B, *Scytonema mirabile*; C, *S. ocellatum*; D, *Fischerella epiphytica*.
Source: Geitler (1932).

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20

Taxonomy of cysts

K. Matsuoka and Y. Fukuyo

20.1 Descriptive terminology of dinoflagellate cysts

Some dinoflagellates produce two different types of non-motile cell in their life cycles, referred to as temporary cysts and resting cysts or hypnozygotes (Fig. 6.1). The resting cysts can survive harsh environmental conditions (see Chapter 6) and thus play an important ecological role as the source of inoculum cells for recurrent blooms. They also facilitiate the expansion of the geographical distribution of a species through cyst dispersal. Currently more than 80 marine and 15 freshwater species of modern dinoflagellates are known to produce resting cysts (Table 20.1). The number of the cyst-producing species is small compared with the total number of extant dinoflagellates (more than 2,000).

Table 20.1 Species producing resting cysts in modern dinoflagellates

Species	Reference
Marine	
Prorocentrales	
Prorocentrum lima	Faust (1990)
Prorocentrum marinum	Faust (1993)
Prorocentrum pyrenoideum	Bursa (1959)
Dinophysiales	
Dinophysis acuta	Moita and Sampayo (1993)
Dinophysis tripos	Moita and Sampayo (1993)
Gymnodiniales	
Amphidinium carterae	Cao Vien (1967)
Cochlodinium sp.	Fukuyo (1982)
Cochlodinium sp.	Matsuoka (1985*a*, 1987*a*)
*Gymnodinium catenatum**	Anderson *et al.* (1988); Matsuoka (1987*a*): Hallegraeff *et al*. (1989)
Gymnodinium sp. 1 (= *Gymnodinium microreticulatum*)	Bolch and Hallegraeff (1990)
Gyrodinium (= Gymnodinium) impudicum	Kobayashi *et al*. (2001)

…/…

Species	Reference
Gyrodinium instriatum	Wall and Dale (1968*a*); Fukuyo (1982); Matsuoka (1985*a*); Kojima and Kobayashi (1992)
Gyrodinium resplendens	Dale (1983)
Gyrodinium uncatenum	Tyler *et al.* (1982)
Gyrodinium sp. 1 (= *Gymnodinium aureolum*)	Bolch and Hallegraeff (1990)
Pheopolykrikos hartmannii	Fukuyo (1982); Matsuoka and Fukuyo (1986)
Polykrikos kofoidii	Matsuoka (1985*a*): Morey-Gaines and Ruse (1980); Fukuyo and Matsuoka (1983)
Polykrikos schwartzii	Wall and Dale (1968*a*); Matsuoka (1985*a*)
Woloszynskia sp. 1	Bolch and Hallegraeff (1990)
Katodinium fungiforme	Spero and Moree (1981)
Pfiesteria piscicida	Burkholder *et al*. (1992)
Gonyaulacales	
Bitectatodinium tepikiense	Lewis *et al*. (2001)
Gonyaulax digitalis	Wall and Dale (1968*a*)
Gonyaulax scrippsae	Wall and Dale (1968*a*); Matsuoka (1984*b*)
*Gonyaulax spinifera**	Wall and Dale (1968*a*)
Gonyaulax cf. *spinifera*	Dale (1983)
Gonyaulax verior	Matsuoka *et al*. (1988)
Gonyaulax sp.	Dobell and Taylor (1981)
*Lingulodinium polyedrum**	Wall and Dale (1968*a*); Kobayashi *et al*. (1981); Lewis (1988)
Protoceratium reticulatum	Wall and Dale (1968*a*)
*Alexandrium affine**	Fukuyo *et al*. (1985): Hallegraeff *et al*. (1991)
*Alexandrium catenella**	Yoshimatsu (1981); Fukuyo (1985)
Alexandrium cohorticula	Fukuyo *et al*. (1988)
*Alexandrium fundyense**	McKenzie *et al*. (1998)
Alexandrium globosum	Dale (1977b)
Alexandrium hiranoi	Kita *et al.* (1985)
Alexandrium leei	Fukuyo *et al*. (1988)
Alexandrium lusitanicum	Blanco (1989)
*Alexandrium minutum**	Bolch *et al*. (1991)
*Alexandrium monilatum**	Walker and Steidinger (1979)
Alexandrium pervianum	Fukuyo *et al*. (unpublished data)
Alexandrium pseudogonyaulax	Montresor *et al.* (1993*a*)

…/…

Species	Reference
*Alexandrium tamarense**	Dale (1977*b*); Fukuyo (1985)
Helgolandium subglobosum	von Stosch (1969*b*)
Pyrodinium bahamense var. *bahamense*	Wall and Dale (1969)
Pyrodinium bahamense var. *compressum**	Steidinger *et al*. (1980); Matsuoka *et al*. (1989); Corrales *et al*. (1995)
Pyrophacus horologium	Wall and Dale (1971)
Pyrophacus steinii	Wall and Dale (1971); Matsuoka (1985*b*)
Peridiniales	
Scrippsiella crystallina	Lewis (1991); Ishikawa and Taniguchi (1993)
Scrippsiella lachrymosa	Lewis (1991)
Scrippsiella minima	Gao and Dodge (1991)
Scrippsiella patagonica	Akselman and Keupp (1990)
Scrippsiella precaria	Montresor and Zingone (1988); Ishikawa and Taniguchi (1993); Kobayashi *et al*. (1994)
Scrippsiella ramonii	Montresor (1995)
Scrippsiella rotunda	Lewis (1991); Ishikawa and Taniguchi (1993)
Scrippsiella trifida	Lewis (1991)
*Scrippsiella trochoidea**	Wall *et al*. (1970); Bolch and Hallegraeff (1990); Lewis (1991)
Scrippsiella sweeneyae	Wall and Dale (1968*b*)
Ensiculifera cf. *mexicana*	Wall and Dale (1968*b*)
Ensiculifera carinata	Matsuoka *et al*. (1990)
Pentapharsodinium dalei	Dale (1977*a*, 1978); Lewis (1991)
Pentapharsodinium tyrrhenicum	Montresor *et al*. (1993b)
Peridinium hangoei	Iwasaki (1969): Lewis *et al.* (1984)
Cachonina hallii	von Stosch (1969a)
Coolia monotis	Faust (1992)
Heterocapsa triquetra	Braarud and Pappas (1951)
Protoperidinium acromaticum	Popovsky and Pfiester (1990)
Protoperidinium americanum	Lewis and Dodge (1987)
Protoperidinium avellanum	Wall and Dale (1968*a*); Matsuoka (1984*a*); Lewis *et al*. (1984)
Protoperidinium antarcticum	Akselman (1987)
Protoperidinium brochii	Blanco (1989)

…/…

Species	Reference
Protoperidinium claudicans	Wall and Dale (1968*a*); Akselman (1987)
Protoperidinium compressum	Wall and Dale (1968*a*)
Protoperidinium conicoides	Wall and Dale (1968*a*); Akselman (1987)
Protoperidinium conicum	Wall and Dale (1968*a*); Fukuyo (1980); Kobayashi and Matsuoka (1984); Bolch and Hallegraeff (1990)
Protoperidinium denticulatum	Wall and Dale (1968*a*)
Protoperidinium divaricatum	Matsuoka *et al*. (1982)
Protoperidinium excentricum	Wall and Dale (1968*a*); Lewis *et al*. (1984); Akselman (1987)
Protoperidinium expansum	Hallegraeff and Bolch (1992)
Protoperidinium grandii	Meunier (1910)
Protoperidinium latissinum	Wall and Dale (1968*a*)
Protoperidinium leonis	Wall and Dale (1968*a*)
Protoperidinium minutum	Wall and Dale (1968*a*); Fukuyo *et al*. (1977)
Protoperidinium nudum	Wall and Dale (1968*a*)
Protoperidinium oblongum	Wall and Dale (1968*a*); Akselman (1987); Bolch and Hallegraeff (1990)
Protoperidinium obutsum	Akselman (1987)
Protoperidinium pentagonum	Wall and Dale (1968*a*); Matsuoka (1982); Lewis *et al*. (1984); Inoue (1990)
Protoperidinium punctulatum	Wall and Dale (1968*a*)
Protoperidinium subinerme	Wall and Dale (1968*a*)
Protoperidinium thorianum	Lewis *et al*. (1984)
Protoperidinium thulesense	Dodge (1985)
Protoperidinium cf. *divergens*	Dale (1983)
Protoperidinium sp.	Dale (1983)
Diplopelta parva	Matsuoka (1988); Bolch and Hallegraeff (1990)
Diplopelta symmetrica	Dale *et al*. (1993)
Diplopsalis lenticula	Wall and Dale (1968*a*)
Diplopsalis lebourae	Matsuoka (1988)
Diplopsalopis orbicularis	Wall and Dale (1968*a*); Matsuoka (1988)
Diplopsalopsis latipeltata	Dale *et al.* (1993)
Goitus abei	Matsuoka (1988)
Oblea rotunda	Lewis (1990)

…/…

Species	Reference
Zygabikodinium lenticulatum	Wall and Dale (1968*a*); Akselman (1987); Matsuoka (1988); Bolch and Hallegraeff (1990)
* Harmful species.	
Freshwater species	
Gymnodinium chiastosporum	Cridland (1958)
Gymnodinium dodgei	Sarma and Shyam (1974)
Gymnodinium fungiforme	Biecheler (1952)
Gymnodinium fuscum	Bourelly (1970)
Gymnodinium helveticum f. *achroum*	Skuja (1948)
Gymnodinium impatiens	Skuja (1964)
Gymnodinium paradoxum	von Stosch (1972)
Gymnodinium pseudopalustre	von Stosch (1973)
Cystodinium bataviesnse	Pfiester and Lynch (1980)
Cystodinium cornifax	Schilling (1891)
Ceratium carolinianum	Wall and Evitt (1975)
Ceratium cornutum	von Stosch (1972)
Ceratium furcoides	Hickel (1988)
Ceratium hirundinella	Chapmann *et al.* (1982)
Ceratium rhomboides	Hickel (1988)
Glenodinium emaginatum	Klebs (1912)
Glenodinium montanum	Kelley and Pfiester (1989)
Diplopsalis acuta	Schiller (1937)
Peridinium aciculiferum	Popvsky and Pfiester (1990)
Peridinium bips f. *occulatum*	Park and Hayashi (1993)
Peridinium cinctum f. *ovoplanum*	Pfiester (1975)
Peridinium cinctum f. *westii*	Eren (1969)
Peridinium cunningtonii	Sako *et al*. (1984)
Peridinium gatunense	Pfiester (1977)
Peridinium imconspicuum	Pfiester *et al.* (1984)
Peridinium limbatum	Wall and Dale (1968*a*)
Peridinium lubiniensiforme	Dilwald (1937)
Peridinium penardii	Sako *et al*. (1987)
Peridinium polonicum	Sako *et al.* (1986)
Peridinium volzii	Pfiester and Skvarla (1979)
Peridinium willei	Pfiester (1976)
Peridinium wisconsinense	Evitt and Wall (1968)
Woloszynskia apiculata	von Stosch (1973)
Woloszynskia cestocoetes	Thompson (1950)
Woloszynskia coronata	Woloszynska (1917)
Woloszynskia reticulata	Thompson (1950)
Woloszynskia tenuissima	Woloszynska (1917)
Woloszynskia tylota	Bibby and Dodge (1972)

The important morphological characters for identification of resting cysts are the shape of the cyst body and its ornamentation, wall structure and colour, and the type of aperture or archeopyle through which germinating cells leave the cyst. Archeopyle type can be a very useful character in determining the higher classification rank (genus and family) of cyst species. However, as cysts possess no archeopyle before excystment, it is impossible to use this feature for routine identification. In comparison to the morphology of motile forms, cysts usually have a relatively simple, mostly spherical to peridinioid shape. As a result, identification of cysts based on a single morphological character is not always reliable, and other characters such as the type of archeopyle, morphology of ornaments, wall structure, wall colour and paratabulation are indispensable for classification.

Those who are interested in modern dinoflagellate cysts often have to refer to original descriptions given by palaeontologists who used fossil cyst terminology. These terms are useful not only for palaeontologists but also modern planktologists. Fensome *et al*. (1993) try to unify the classification systems proposed by palaeontologists for fossil cysts and by planktologists for modern motile forms. It is of great value to use common terms for both fossil and modern cyst forms.

20.1.1 Morphology of the cyst body

Dinoflagellate cysts are classified into three major groups based on the position of their formation within the planozygote, the life-history stage that develops into the hypnozygote (resting cyst) (Fig. 20.1).

The proximate cyst is formed directly beneath the theca of the planozygote and therefore its volume occupies approximately one-half to one-third of the original

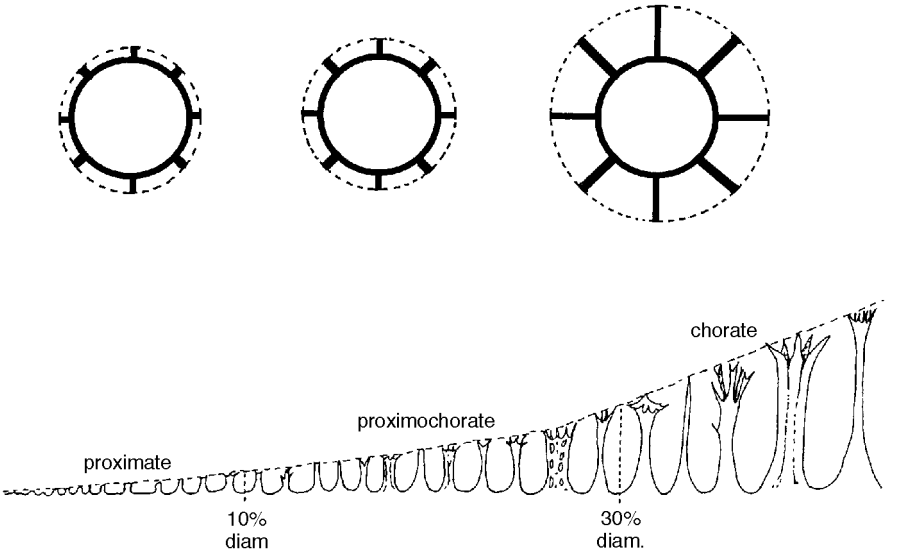

Figure 20.1
Major cyst shapes: proximate, proximochorate and chorate cyst.
Source: modified from Sarjeant, 1984.

cell. Some cysts of this type have a characteristic ornamentation that possibly reflects the original plate, cingulum, sulcus and other thecal structures such as the apical groove of the motile forms. Proximate cyst bodies vary from spherical to peridinioid and sometimes have several projections on the surface.

The chorate cyst is characterized by various kinds of ornaments ('processes') rising from the cyst surface. These ornaments functionally support the cell wall of the planozygote from the cyst surface and are morphologically variable. Process formation during maturation from planozygote to hypnozygote has been observed in *Lingulodinium polyedrum* by Kokinos and Anderson (1995) and in *Spiniferites ramosus* and *S. membranaceus* by Lewis *et al*., 1999). Generally the cyst body of this type is spherical, subspherical or ovoidal. The volume of the cyst cavity is reduced compared with the planozygote, mostly less than one-third of the original.

The cavate cyst consists of more than two walls clearly separated, and usually possesses a cavity partly or entirely around the cyst body. Therefore, the outline of the cyst is variable. The volume of the cyst containing the protoplasm is much reduced during maturation from planozygote to hypnozygote, and the inner cyst body is probably less than one-fifth of the original in volume.

In general, the outlines of modern dinoflagellate cysts are relatively simple in comparison with fossil forms; that is, they are mainly spherical, subspherical, ovoidal, ellipsoidal or peridinioid. Some brackish water cysts, however, vary in shape from simply spherical through peridinioid to cursiform. It is notable that even in a single species, environmental parameters such as salinity, temperature and nutrients can produce remarkable variation in the cyst shape. This is well investigated for *Spiniferites cursiformis* Wall and Dale and *Tectatodinium pellitum* Wall and Dale found in Quaternary sediments of the Black Sea (Wall *et al*., 1973).

20.1.2 Wall structure and colour

The wall of modern cysts consists of one, two or three layers composed mostly of biopolymers, chemically similar to the sporopollenin of spores and pollen grains in higher plants (Kokinos *et al*., 1998), and sometimes calcium carbonate (e.g. *Scrippsiella)*. The cyst wall can be composed of up to three or four layers (Evitt, 1985) termed the autophragm, periphragm, mesophragm and endophragm (Fig. 20.2).

The colour of the cyst wall is also variable; it can be mostly transparent, light yellow, pale brown, brown or dark brown. Living cysts partly composed of calcium carbonate such as *Scrippsiella trochoidea* are dark brown to black.

20.1.3 Morphology of surface ornamentation

For the description of surface ornaments of cysts, the terminology for pollen grains and spores is adopted. Some terms frequently used for modern cysts are shown in Fig. 20.3.

20.1.4 Archeopyle

The term 'archeopyle' is defined by Evitt (1963) as an excystment opening formed at the germination stage of dinoflagellate cysts. In dinoflagellate cysts, apical, intercalary, precingular, hypocystal archeopyle types, and combinations of these series have been recognized. However, it is not possible to use these definitions for cysts of

gymnodinialean species, which are not covered with typical thecal plates at the motile stage. Matsuoka (1985*a*) proposed new descriptive terms for the archeopyle of modern dinoflagellate cysts in both naked and thecate dinoflagellates, and divided them into saphopylic, theropylic and cryptopylic archeopyles (Fig. 20.4).

20.1.4.1 *Saphopylic archeopyle*

Archeopyle sutures correspond to paraplate boundaries and the operculum. Part of the cyst wall corresponding to the archeopyle is always detached from the cyst body. The archeopyle type can be subdivided into either apical, intercalary, precingular, 'hypocystal' or a combination from one or more plate series. Most modern cysts belonging to the Peridiniales and Gonyaulacales have this archeopyle type.

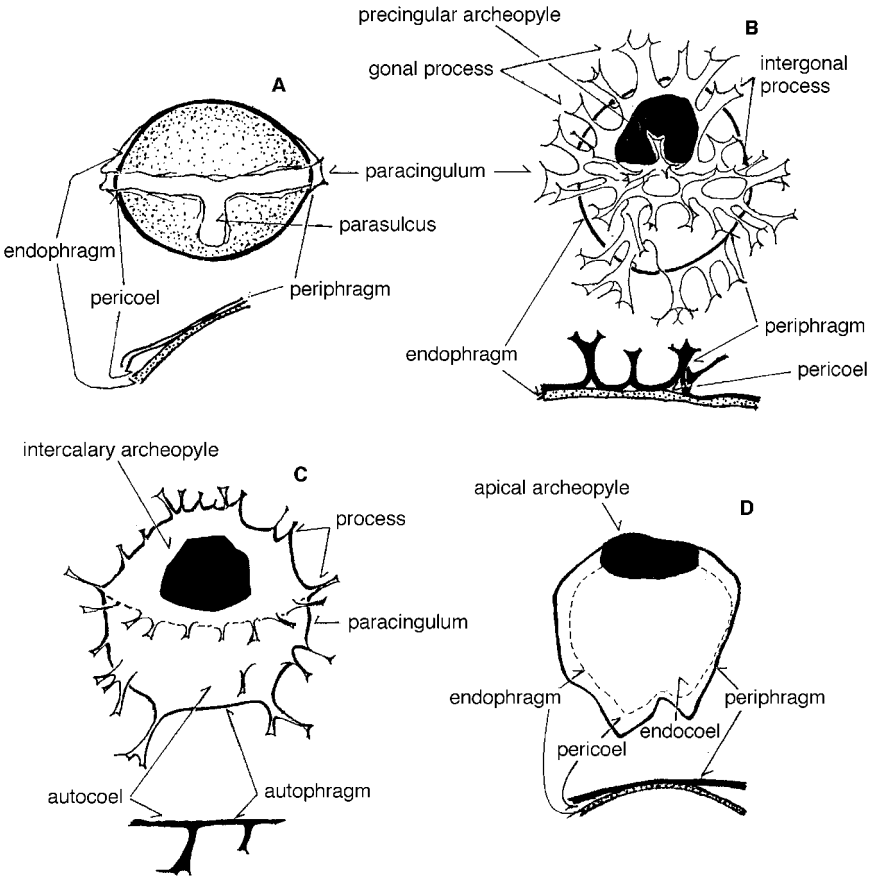

Figure 20.2
Wall structure of modern cysts and descriptive terms. A, proximate cyst with two layers; B, chorate cyst with two layers and processes; C, proximochorate cyst with single layer; D, proximate cyst with two layers.

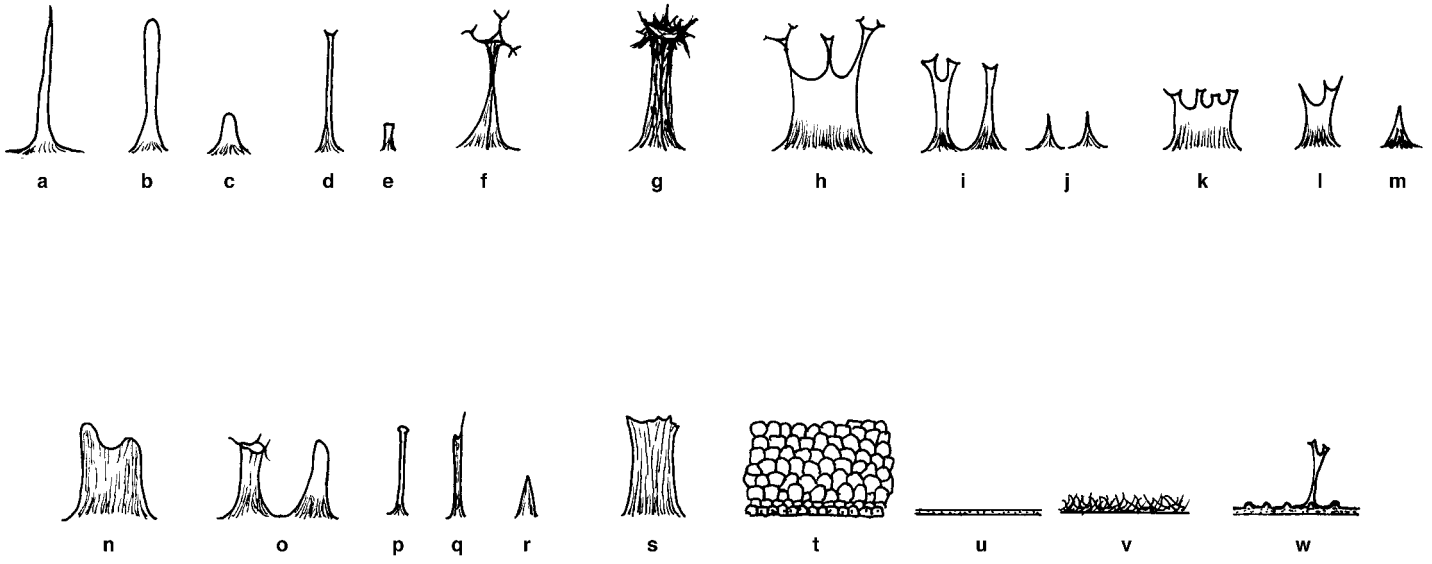

Figure 20.3
Descriptive terms for cyst wall and processes of modern dinoflagellate cysts. A, acuminate (*Lingulodinium machaerophorum*); B, bulbous (*Lingulodinium machaerophorum*); C, excavate (*Lingulodinium machaerophorum*); D, capitate (cyst of *Protoceratium reticulatum*); E, cylindrical (cyst of *Protoceratium reticulatum*); F, trifurcate (*Spiniferites* spp.); G, orthogonal (*Polykrikos kofoidii*); H, vallate (*Spiniferitis mirabilis*); I, oblate (*Polysphaeridium zoharyi*); J, conical (*Selenopemphix quanta*); K, hysttricate (*Spiniferites mirabilis*); L, antlerform (*Spiniferites mirabilis*); M. conical with striations at the proximal base (*pheopolykrikos hartmanii*); N, machicolate (*Cochlodinium* cf. *polykrikoides*); O, patulate and excavate (*Protoperidinium* sp.); P, capitate (*Islandinium minutum* var. *cezare*); Q, cylindrical (*Scrippsiella trochoidea* calcareous spinose cyst); R, conical (*Scrippsiella* sp.); S, membranous (*Spiniferites membranaceus*; T, microreticulate (*Gymnodinium catenatum*); U, psilate (*Alexandrium tamarense*); V, fibrous (*Bitectatodinium tepikiense*); W, scabrate (*Polysphaeridium zoharyi*).

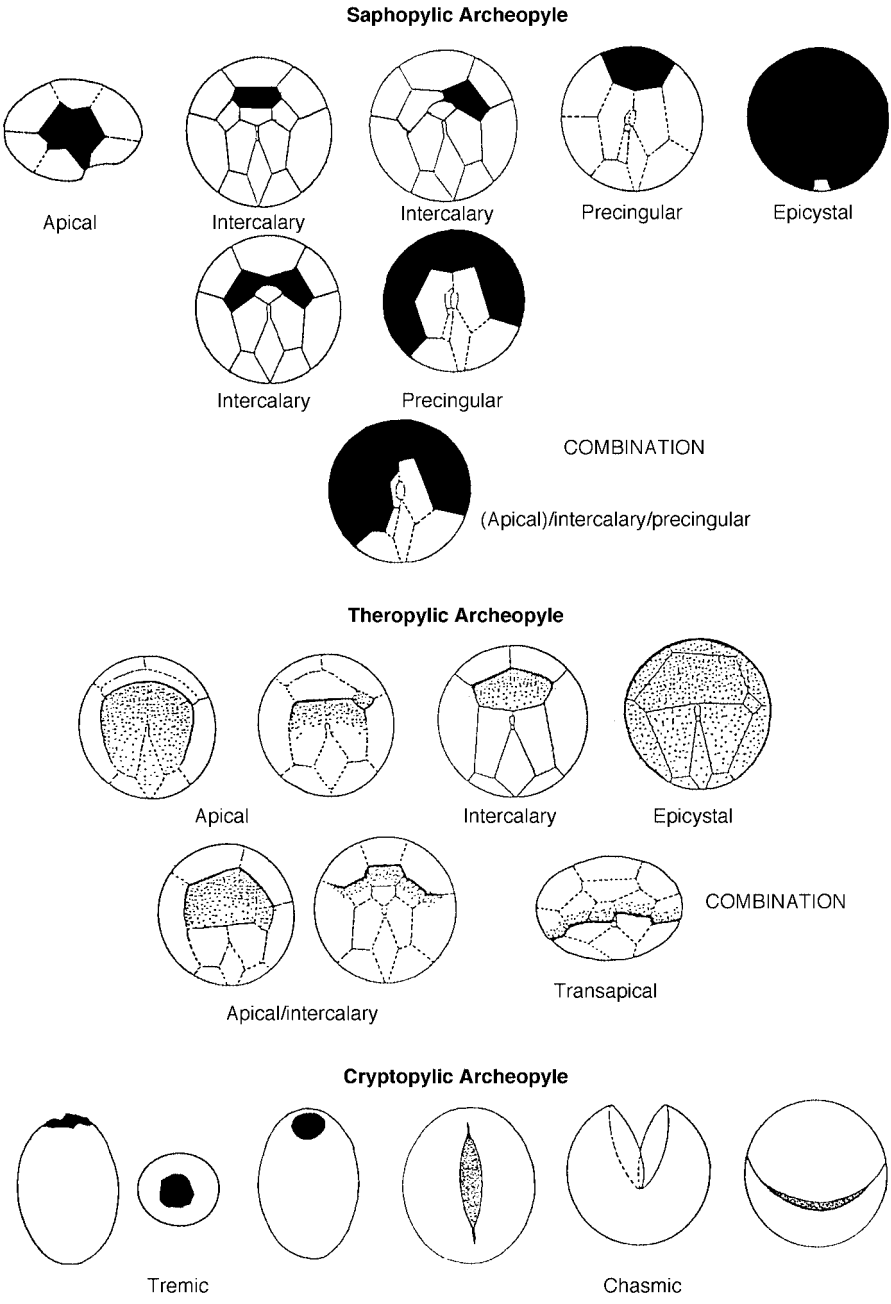

Figure 20.4
Archeopyle types in modern dinoflagellate cysts.

20.1.4.2 *Theropylic archeopyle*

Archeopyle sutures follow paraplate boundaries, but the operculum is usually attached to the cyst. This is caused by incomplete development of archeopyle structures on the cyst body. This archeopyle can also be subdivided into several types on the basis of the position of the sutures. Matsuoka *et al*. (1989) showed a provisional subdivision for this archeopyle, but more careful examination is needed to confirm this. Modern cysts produced by diplopsalid and calciodinellid species have this archeopyle type.

20.1.4.3 *Cryptopylic archeopyle*

The archeopyle suture does not reflect any plate boundary and the operculum is detached or free from the cyst. This is because the motile forms have no thecal plate. Some modern gymnodinialian and gonyaulacacean cysts have this archeopyle type. On the basis of the shape of the opening, this archeopyle type is classified into two forms, chasmic (slit-like opening) and tremic (hole-like opening) (Matsuoka, 1985*a*).

It is notable that some modern cysts do not show any distinct excystment aperture after germination, probably because of their fragile cyst wall. This archeopyle type includes most cysts of *Alexandrium* species, *Pentapharsodinium faeroense* Indelicato and Loeblich, and *Ensiculifera imariense* Kobayashi and Matsuoka.

20.2 Description of harmful marine dinoflagellate cysts

20.2.1 Prorocentrales cysts

Only two species of the *Prorocentrales* producing cysts have been observed. These are *P. lima* and *P. marinum* (Faust, 1990, 1993). These cysts are morphologically simple, spherical and similar to each other. As there have been no records in modern sediments, these cysts probably do not fossilize.

20.2.2 Dinophysiales cysts

Sexuality of *D.* cf. *acuminata* has been documented on the basis of the presence of planozygotes possessing two trailing flagella by McLachlan (1993), but there is no evidence of hypnozygotes or resting cysts for this species. Two other species, *D. acuta* and *D. tripos*, have been observed by Moita and Sampayo (1993) to produce resting cysts. These cysts have not yet been fully studied for their morphology including wall and archeopyle structures, and have never been recorded from modern sediments.

20.2.3 Gymnodinalian cysts (Pl. 20.1)

The shape of the cyst body is mostly spherical to ovoidal and sometimes ellipsoidal, with or without spinate or reticulate ornaments on the surface. The cyst wall is organic and pale brown, brown, rarely reddish-brown in colour; mostly composed of a single layer and sometimes two layers. The archeopyle type is cryptopylic, chasmic or tremic.

20.2.3.1 *Toxic species producing cysts*

Gymnodinium catenatum Graham; possible ichthyo-toxic species producing cysts; *Cochlodinium* sp. cf. *C. polykrikoides* Margalef.

20.2.4 Gonyaulacalean cysts (Pl. 20.2)

The shape of the cyst body is basically spherical to ellipsoidal and rarely discoidal, and sometimes with or without process-like ornaments. The cyst wall is organic, colourless and sometimes transparent; in some forms is composed of a single or, more often, two layers. The archeopyle type is mostly saphopylic, precingular, but sometimes epicystal or combination. In the cysts of *Alexandrium* spp. and *Gonyaulax verior*, no typical archeopyle is formed.

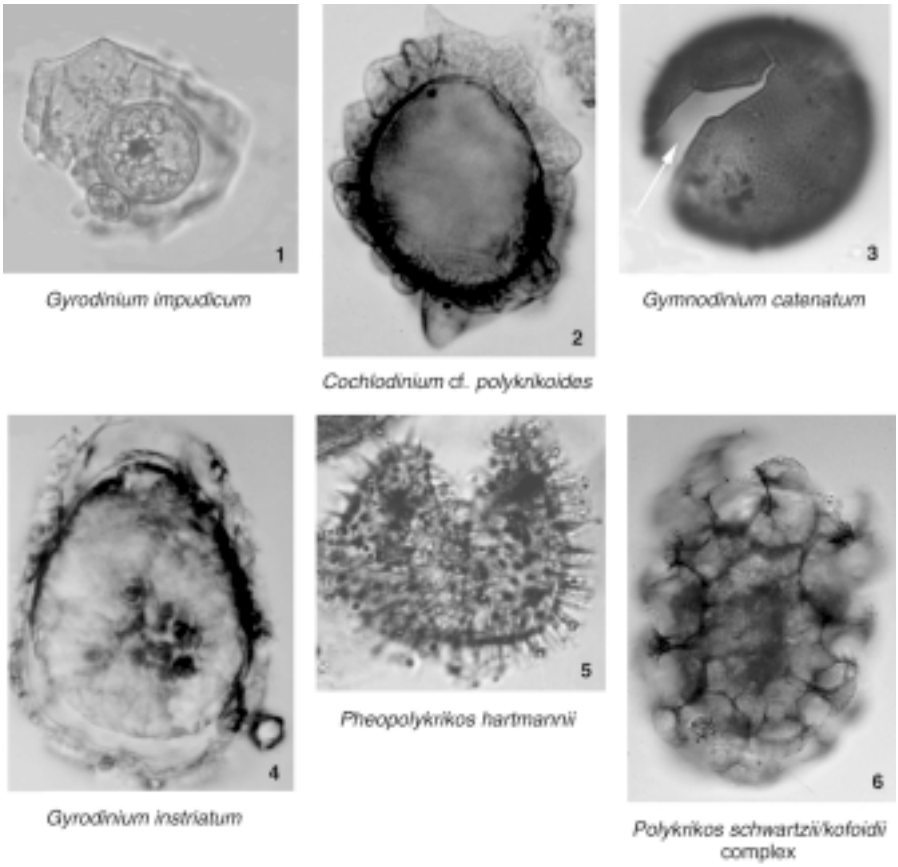

Plate 20.1
Cysts of Gymnodiniales.

20.2.4.1 *Toxic species producing cysts*

Lingulodinium polyedrum (Stein) Dodge; *Protoceratium reticulatum* (Claparéde and Lachmann) Bütschli; *Alexandrium affine* (Inoue and Fukuyo) Balech; *Alexandrium andersonii* Balech; *Alexandrium catenella* (Whedon and Kofoid) Balech; *Alexandrium fundyense* Balech; *Alexandrium minutum* Halim; *Alexandrium tamarense*

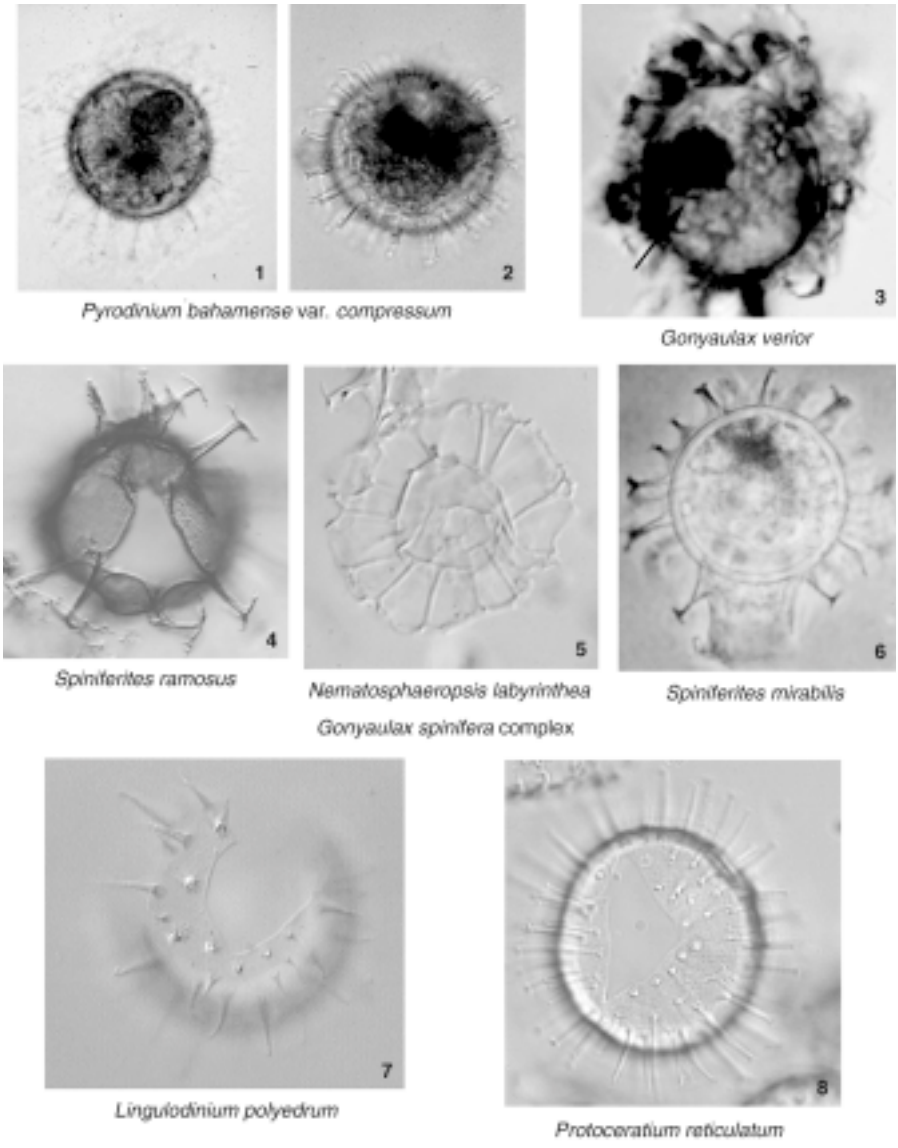

Plate 20.2
Cysts of Gonyaulacales.

(Lebour) Balech; *Pyrodinium bahamense* Plate var. *compressum* (Böhm) Steidinger, Tester and Taylor.

20.2.5 Peridinialean cysts (Pl. 20.3)

The shape of the cyst body is mainly spherical, ellipsoidal, peridinioid, and rarely discoidal, mainly without process-like ornaments. The cyst wall is mainly organic

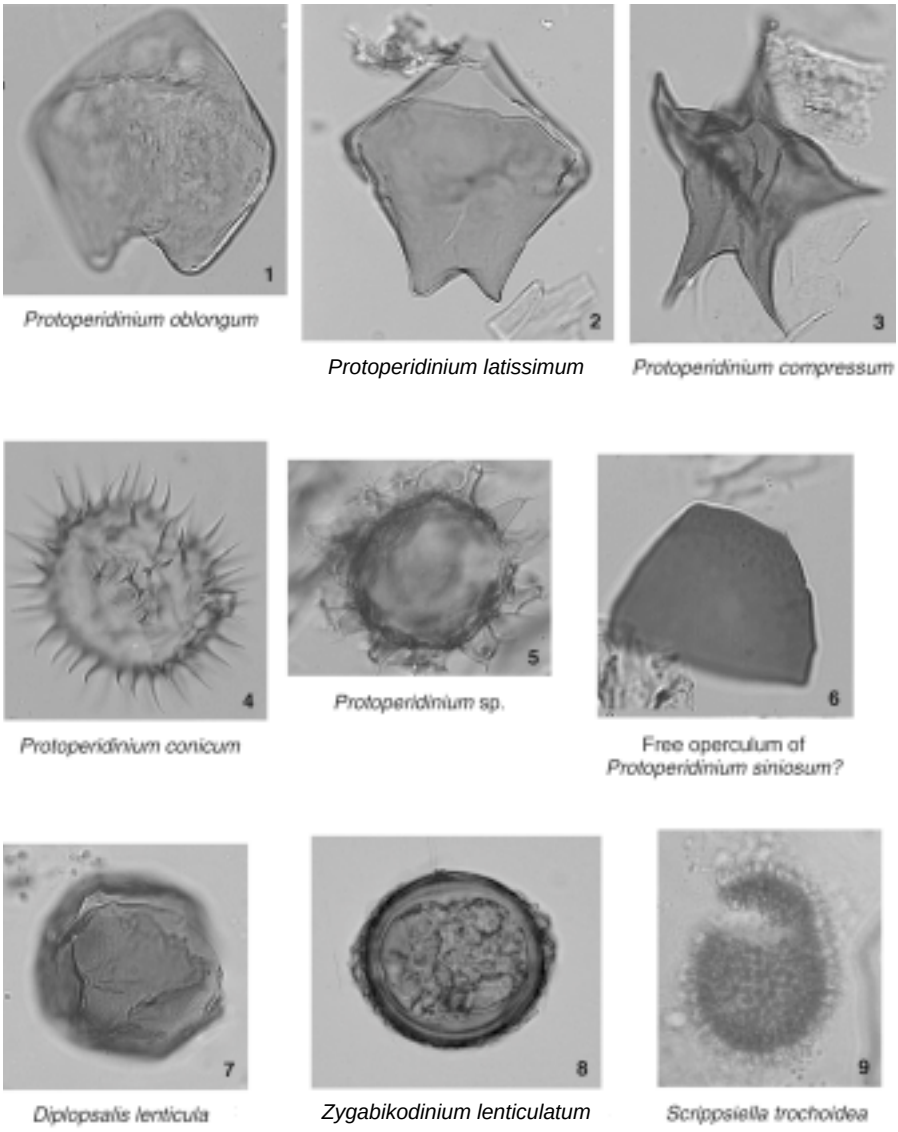

Plate 20.3
Cysts of Peridiniales.

and mostly brown in colour, rarely transparent, and sometimes calcareous; mainly composed of a single and rarely two layers. The archeopyle type is mainly saphopylic, of the intercalary and sometimes theropylic type, or apical, intercalary, epicystal and combination types.

20.2.5.1 *Harmful species producing cysts*

Scrippsiella trochoidea (Stein) Loeblich, III.

20.3 Description of dinoflagellate cysts of harmful species

20.3.1 Cysts of Gymnodiniales

20.3.1.1 *Gymnodinium catenatum* Graham (Pl. 20.1, Fig. 3; Pl. 20.4, Figs. 5–8)

- *Gymnodinium catenatum* (Graham, 1943, pp. 259–61, Figs. 1, 2;
- Cyst form: *Gymnodinium catenatum* (Anderson *et al.*, 1988, pp. 255–62, Figs. 1–16).

Shape: proximate, spherical without horns and spines. *Size*: small to intermediate, 45–63 µm in diameter. *Wall structure and colour*: autophragm; microreticulate ornaments which probably reflect the pattern of amphiesmal vesicles on motile stage. Shape and size of each reticulation is variable. Dark brownish to reddish colour. *Paracingulum*: two rows of relatively smaller paravesicles reflect the cingulum of the motile cell. *Parasulcus*: linear arrangement of smaller and larger reticulations shows the sulcus of the motile cell. *Archeopyle and operculum*: cryptophylic, chasmic type; slit opening encircling over the cell diameter; operculum adnate. *Other features*: Smaller paravesicles going up from the paracingulum to the apex and

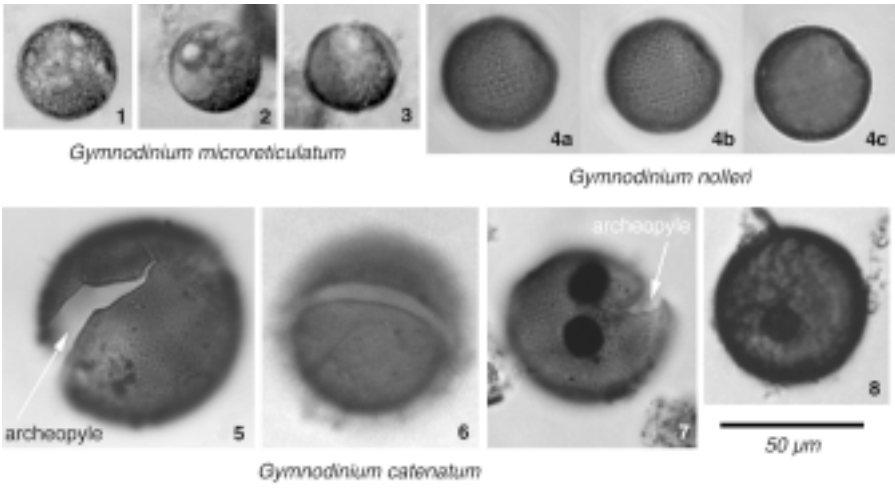

Plate 20.4
Cysts of *Gymnodinium catenatum* and allied species.

then encircling around the apex reflect the apical groove of the motile cell. The living cyst is dark brown and contains many food reserves of starch and oil drops, and a single red-pigmented body.

Comment: The cyst with microreticulate surface ornamentation and chasmic archeopyle are also found in non-toxic *Gymnodinium nolleri* (Pl. 20.4, Figs. 4a–4c) and *Gymnodinium microreticulatum* (Pl. 20.4, Figs. 1–3). According to Bolch *et al*. (1999), a characteristic useful for distinguishing these cysts from each other is the diameter; the cyst of *G. catenatum* being the largest, 36–62 µm, *G. nolleri* 28–38 µm, and the smallest *G. microreticulatum* 17–28 µm. The chasmic archeopyle along the parasulcus of *G. microreticulatum* is different from others. When the cyst is filled with fresh protoplasm, it is also very difficult to distinguish this from other round brown cysts belonging to *Brigantedinium*, and some cysts of the diplopsalid group.

20.3.1.2 *Cochlodinium* sp. cf. C. *polykrikoides* (Margalef, Pl. 20.1, Fig. 2)

- *Cochlodinium polykrikoides* (Margalef, 1961, pp. 76, 78, Fig. 27m;
- Cyst form: cyst of *Cochlodinim* sp. Fukuyo, p. 207, Pl. II, Figs. 1–4);
- Cyst of *Cochlodinim* sp. Matsuoka and Lee, Pl. 5, Fig. 2).

Shape: chorate; subspherical, ovoidal, or ellepisoidal. *Size*: intermediate, 25–33 µm × 38–40 µm in diameter. *Wall structure and colour*: periphragm and endophragm strongly adpressed except for processes or ornaments; granular on surface; dark brownish. Ornament: machichorate (membranous), or slender cylindrical to capitate with closed distal extremities; up to 7 µm in length. *Archeopyle and operculum*: cryptopylic, chasmic type.

Comment: The cyst-motile form relationship of this species is not fully understood at this time. Two incubation experiments have been carried out by Fukuyo (1982) and Matsuoka and Lee (1994). The cysts provided by Fukuyo (1982) were ellipsoidal covered with machichorate ornaments on the surface and those of Matsuoka and Lee (1994) were subspherical to ovoidal with slender cyclindrical processes. From both experiments, a single germinated cell was similar to a solitary cell of *Cochlodinium polykrikoides*, however these two experiments could not confirm a typical chain form of *C. polykrikoides*. From the surface sediments where a dense bloom of *C. polykrikoides* occurred on the Pacific coast of Guatemala in 1995, abundant ellipsoidal cysts covered with machichorate ornaments were found (Sosales-Loessener *et al*., 1996).

20.3.2 Cysts of Gonyaulacales

20.3.2.1 *Lingulodinium polyedrum* (Stein) Dodge (Pl. 20.2, Fig. 7)

- *Gonyaulax polyedra* (Stein, 1883, p. 13, Pl. 4, Figs. 7–9);
- Synonym: *Lingulodinium polyedrum* (Stein) (Dodge, 1989, p. 291, Figs. 1H, I, 34–38);
- Cyst from: *Hystrichosphaeridium machaerophorum* (Deflandre and Cookson, 1955, p. 274, Pl. 9, Figs. 4, 8);
- Synonym: *Lingulodinium machaeophorum* (Deflandre and Cookson) Wall, 1967, pp. 109–10, Pl. 15, Figs. 16, 17.

Shape: chorate; spherical with neither apical nor antapical bosses. As a large archeopyle is formed at germination, the shape is sometimes hemispherical. *Size*: small to intermediate, 35–50 µm in diameter. *Wall structure and colour*: periphragm

and endophragm strongly adpressed except for processes; granular on surface; colourless and transparent. *Processes*: intratabular; variable in shape, hollow, and excavate, bulbous to acuminate with closed distal extremities; up to 17 µm in length. *Archeopyle and operculum*: saphopylic, combination precingular type formed by loss of three to four precingular and sometimes two additional anterior intercalary paraplates; operculum free. *Other features*: Important morphological characters for this species are a large spherical or hemispherical cyst body and hollow and excavate, bullbose to acuminate processes. The processes are variable in shape and length; rarely, nodular forms are observed.

Comment: *Lingulodinium machaerophorum* (Deflandre and Cookson) is the palaeontological name for this cyst. In surface sediments of Australian coastal waters, hemispherical specimens formed by loss of the whole epicyst were described as *Lingulodinium hemiscystum* by McMinn (1990). However, as the motile form is not yet known from an incubation experiment with living cysts, and as other morphological features of this cyst except for its epicystal archeopyle are similar, *L. hemicystum* is probably conspecific to *L. machaerophorum.*

20.3.2.2 *Protoceratium reticulatum* (Claparéde and Lachmann) Bütschli (Pl. 20.2, Fig. 8)

- *Peridinium reticulatum* (Claparéde and Lachmann, 1859, p. 405, Pl. 20, Fig. 3)
- Synonym: *Protoceratium reticulatum* (Claparéde and Lachmann) (Bütschli, 1885, p. 1007, Pl. 52, Fig. 2). *Gonyaulax grindleyi* (Reineke, 1967, pp. 157–60, Fig. 1)
- Cyst from: *Operculodinium centrocarpum* (Deflandre and Cookson) (Wall, 1967, p. 111, Pl. 16, Figs. 1, 2, 5). Non *Hystrichosphaeridium centrocarpum* (Deflandre and Cookson, 1955, pp. 272–3, Pl. 8, Figs. 3, 4).

Shape: chorate; spherical to subspherical. *Size*: Small to intermediate, 35–45 µm in diameter. *Wall structure and colour*: periphragm and endophragm strongly adpressed except for processes; granular on surface; colourless and transparent. *Processes*: intratabular; slender and sometimes flexuous, hollow, and capitate to short cyclindrical with closed distal extremities; up to 17 µm in length. Archeopyle and operculum: saphopylic, precingular type formed by loss of the third precingular paraplate (3'); operculum free. *Other features*: The processes are variable in shape and length; in blackish water environments, short process or nodular forms are often observed.

Comment: The Miocene fossil species, *Hystrichosphaeridium centrocarpum* is a different cyst form of modern *Protoceratium reticulatum* (Matsuoka *et al*., 1997). Two other modern cysts of *Protoceratium reticulatum* are reported by Wall and Dale (1968*a*); *Operculodinium israelianum* and *O. giganteum*.

20.3.2.3 *Pyrodinium bahamense* Plate *var. compressum* (Böhm) Steidinger, Tester and Taylor (Pl. 20.2, Figs. 1–2)

- *Pyrodinium bahamense* Plate forma *compressa* (Böhm, 1931, p. 191, Fig. 4); *Pyrodinium bahamense* Plate var. *compressa* (Böhm) (Steidinger, Tester and Taylor, 1980, p. 329–34);
- Cyst form: *Hemicystodinium zoharyi* (Rossignol, 1962, pp. 132–3, Pl. 2, Fig. 10);
- Synonym: *Pyrodinium bahamense* Plate var. *compressum* (Matsuoka *et al*., 1989, pp. 301–4).

Shape: chorate; spherical, covered with many processes. *Size*: intermediate, 55–70 µm in diameter. *Wall structure and colour*: periphragm and endophragm strongly adpressd except for processes, and granular surface; colourless. *Processes*: intratabular; long, slender, cylindrical to tubiform, and capitate distal extremities; 6–15 µm in length. *Archeopyle and operculum*: epicystal; operculum composed of all paraplates of the free epicyst, but archeopyle sutures are often observed on the attached operculum. *Other features*: As the development of archeopyle sutures is incomplete, the number of opercular pieces is variable and also the shape of cyst body varies from spherical to hemispherical.

Comment: Another variety in this species, *Pyrodinium bahamense* var. *bahamense* also produces a spherical cyst with many slender processes. The surface ornamention of the cyst of var. *compressum* is coarser than that of var. *bahamense*, but other morphological differences between these two varieties have not yet been fully described. This cyst differs from other spherical cysts with many processes such as *Protoceratium reticulatum* and *Lingulodinium polyedrum* in having a distinct epicystal archeopyle and slender tubiform to cylindrical processes.

20.3.2.4 *Alexandrium andersonii* Balech (Pl. 20.5, Figs. 6–7)

- *Alexandrium andersonii* (Balech, 1990, p. 394, Figs. 34–50);
- Cyst form: *Alexandrium andersonii* Balech; Montresor *et al*.,1990, Fig. 3i.

Shape: proximate; spherical, without any surface ornament. *Size*: small, 15–20 µm in diameter. *Wall structure and colour*: thin periphragm and thick endophragm strongly adpressed without any surface ornament; colourless. *Archeopyle and operculum*: unknown in detail. *Other features*: At present, only a single report on the cyst of this species has been provided (Montresor *et al*., 1990), and further morphological information is not available.

Comments: The cyst of *A. andersonii* is similar to *A. affine*, but it is smaller.

20.3.2.5 *Alexandrium minutum* Halim (Pl. 20.5, Fig. 5)

- *Alexandrium minutum* (Halim, 1960, pp. 102–5, Fig. 1);
- Synonym: *Pyrodinium minutum* (Halim) (Taylor, 1976, p. 13, Pl. 2, Figs. 1–5);
- Cyst form: *Alexandrium minutum* Halim (Bolch *et al*., 1991, p. 218, Figs. 1–2, 8–9).

Shape: proximate; bean-shaped, circular in apical view, and reniform in lateral view, without any surface ornament. *Size*: small, 20–30 µm in diameter. *Wall structure and colour*: thin periphragm and thick endophragm strongly adpressed without any surface ornament; colourless. *Archeopyle and operculum*: possibly chasmic, but unknown in detail. *Other features*: The cyst is usually covered with a transparent gelatinous substance sometimes including fine mineral and detrital particles of diatoms, silicoflagellates and other microplankton. After germination, the empty cyst is rarely preserved in sediment because of its thin and fragile cyst wall. The living cyst contains many colourless food reserves of starch and oil drops, and a single red-pigmented body.

Comments: The cyst of *A. minutum* is similar to *A. lusitanicum*, but it differs in its smaller cyst body (Bolch *et al*., 1991), and also differs from other cysts of *Alexandrium* in being reniform in lateral view.

20.3.2.6 Alexandrium affine (Inoue and Fukuyo) Balech (Pl. 20.5, Figs. 3–4)

- *Alexandrium affine* (Inoue and Fukuyo) (Balech, 1985, p. 38, Fig. 6);

- Synonym: *Protogonyaulax affinis* (Inoue and Fukuyo in Fukuyo *et al*., 1985, p. 30, Figs. 1E, 3A–3C, 24–29).

Shape: spherical without any surface ornament. *Size*: small, 30–35 µm in diameter. *Wall structure and colour*: thin periphragm and thick endophragm strongly adpressed without any surface ornamen; colourless. *Archeopyle and operculum*: unknown in detail. *Other features*: Almost the same as *A. minutum*. The empty cyst is not preserved in sediment because of its thin and fragile periphragm.

Comments: It is very difficult to identify the cyst of *A. affine* in sediments, because of its simple, spherical cyst body with no surface ornamentation.

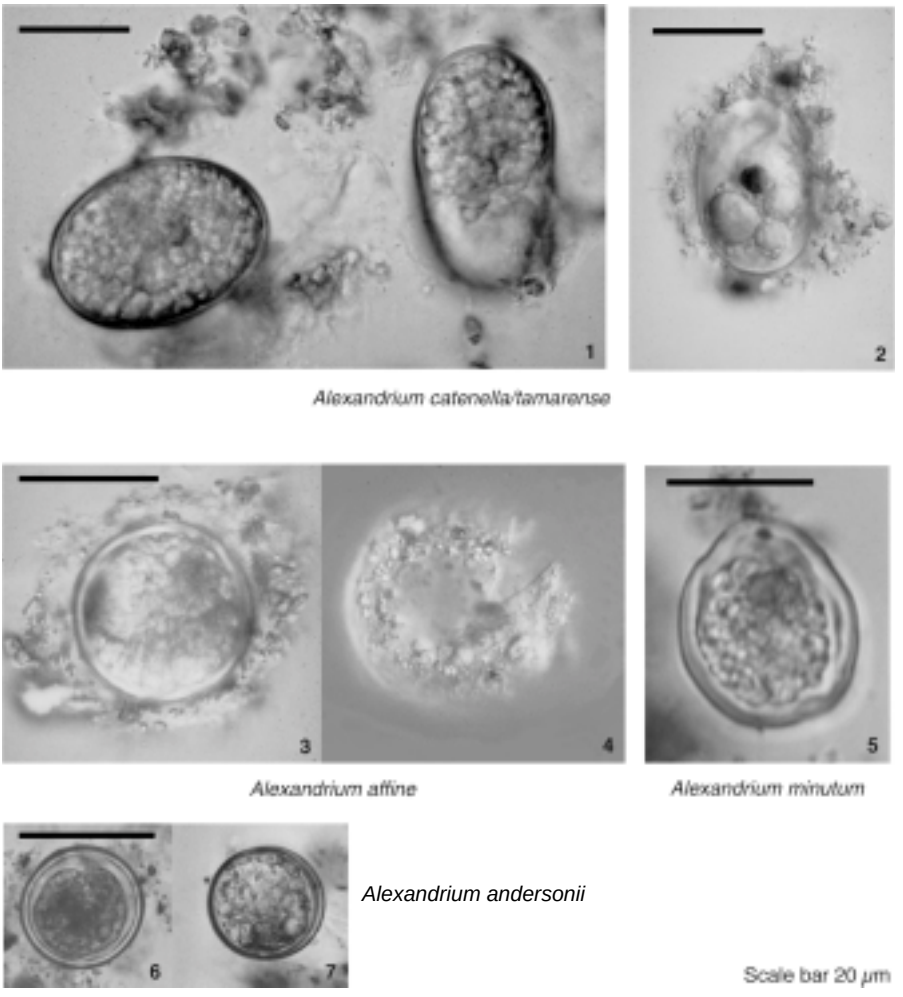

Plate 20.5
Cysts of *Alexandrium*.

20.3.2.7 *Alexandrium catenella* (Whedon and Kofoid) Balech (Pl. 20.5, Figs. 1–2)

- *Alexandrium catenella* (Whedon and Kofoid) (Balech, 1985, p. 37, Fig. 2);
- Synonym: *Gonyaulax catenella* (Whedon and Kofoid, 1936, pp. 25–31, Figs. 1–7, 14. *Alexandrium excavatum* (Braarud) (Balech and Tangen, 1985, p. 338). *Gonyaulax tamarensis* var. *excavata* (Braarud, 1945, pp. 10–11, Pl. 2, Figs. n, o);
- Cyst form: *Protogonyaulax catenella* (Whedon and Kofoid) Taylor or *P. tamarense* (Lebour) Taylor; (Fukuyo, 1985, pp. 531–4, Fig. 2 o–p.).

Shape: proximate; elongate, cylindrical with rounded ends, and without any surface ornament. *Size*: small, 38–56 µm in length, 23-32 µm in width. *Wall structure and colour*: thin periphragm and thick endophragm strongly adpressed without any surface ornament; colourless. *Archeopyle and operculum*: chasmic?, unknown in detail.

Other features: Almost the same as *A. minutum*. The living cyst contains many colourless food reserves of starch and oil drops, and one to two red-pigmented bodies.

Comments: The cyst of *A. catenella* is morphologically identical to *A. tamarense* (Lebour) Balech, and it is impossible to distinguish them from each other on the basis of cyst morphology. Germination experiments are required for this purpose. The cyst is also similar to that of *A. ostenfeldii* except for the cyst diameter.

20.3.3 Cysts of Peridiniales

20.3.3.1 *Scrippsiella trochoidea* (Stein) Loeblich III (Pl. 20.3, Fig. 9)

- *Scrippsiella trochoidea* (Stein) (Loeblich III, 1976, p. 25);
- Synonym: *Glenodinium trochoideum* (Stein, 1883, p. 13, Pl. 3, Figs. 27–9. *Peridinium trochoideum* (Stein) (Lemmermann, 1910, p. 673, Figs. 14–17);
- Cyst form: *Peridinium trochoidea* (Stein) Lemmermann (Wall and Dale, 1968*b*, p. 1401, Pl. 172, Figs. 1–4, 27, text–Fig. 2, 1–3).

Shape: chorate; spherical to ovoidal with many processes. *Size*: small; 25–48 µm in length, 25–44 µm in width. *Wall structure and colour*: thick and calcareous periphragm covered with many spines, and thin and transparent endophragm; dark brown in colour with granular surface. *Processes*: calcareous, nontabular, slender, cylindrical, and solid with capitate distal ends, densely distributed on the surface; length of processes variable. *Archeopyle and operculum*: theropylic; archeopyle suture consisting of three to four sides of paraplate, however its location is not known in detail, probably anterior intercalary/precingular. *Other features*: After chemical treatment with acidic solutions such as HCl, calcareous features of the cyst are removed, however the colourless organic endophragm still remains. Because of its calcareous wall and processes, the living cyst is dark brown to black in colour, and usually contains a single red-pigmented body.

Taxonomic note: Some other species of *Scrippsiella* such as *S. lachrymosa*, *S. trifida*, *S. rotunda*, *S. precaria* and *S. crystallina* also possess calcareous wall and processes. Among them, *S. trochoidea* and *S. precaria* are characterized by long and slender calcareous processes, and are very difficult to distinguish from each other.

20.4 Cysts of other harmful phytoplankton

Some other phytoplankton species belonging to the Raphidophyceae also produce resting cysts in their life cycle. They include *Chattonella antiqua* (Hada) Ono, *C. marina* (Subrahmanyan) Y. Hara and Chihara, *Heterosigma akashiwo* (Hada) Hada and *Fibrocapsa japonica* Toriumi and Takano. The cysts produced by these species are generally small and spherical without any ornamentation on the surface. The morphology of their germination apertures is not fully understood.

20.4.1.1 *Chattonella antiqua* (Hada) Ono (Fig. 20.5A)

- Cyst from: *Chattonella antiqua* (Hada) (Ono and Takano, 1980); (Imai and Itoh, 1988, pp. 36–7, Pl. 1, Figs. A, C, E, G; Pl. 2, Figs. A–D; Pl. 3, Figs. A–D. *Chattonella* sp. Imai and Itoh, 1986, p. 62, Pl. 1, Figs. A–F).

Shape: Hemispherical when the cysts adhere to the solid surface of diatom frustules and sand grains, and sometimes spherical when unattached. *Size*: small; 25–35 µm in diameter, and 15–25 µm in height. *Wall relationship and feature*: probably a single layer and transparent without any ornamentation on surface. *Opening for germination*: circular and *c.* 7 µm in diameter, formed on the top of the cyst, with the cover completely detached after germination. *Other features*: Living cysts filled with fresh protoplasm are yellow-green to brownish in colour, and contain several spots of dark brown or black material. Under a fluorescence microscope, living cysts stained with DAPI show the nucleus in blue-white colour.

Comments: Cysts of *Chattonella marina* (Subrahmanyan) Hara and Chihara have the same morphology as *C. antiqua*.

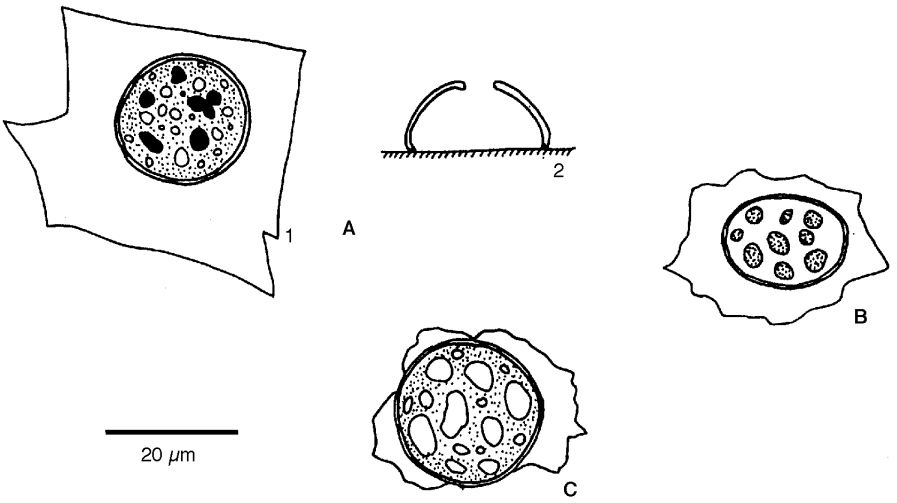

Figure 20.5
Raphidophycean cysts. A, *Chattonella antiqua*: (1) living cysts, attached to a sediment particle; (2) empty cyst with circular opening; B, *Heterosigma akashiwo*, living cyst; C, *Fibrocapsa japonica*.

20.4.1.2 *Heterosigma akashiwo* (Hada) Hada (Fig. 20.5B)

- Cysts of *Heterosigma akashiwo* (Hada) (Hada, 1967); (Imai *et al*., 1993, pp.1669–73).

Shape: spherical, usually covered with mucilage. *Size*: very small; *c*. 10 µm in diameter. *Wall relationship and feature*: probably a single layer and transparent without any surface ornamentation. *Opening for germination*: unknown. *Other features*: Living cysts filled with fresh protoplasm are light yellow-green to brownish in colour. With blue light exitation under a fluorescence microscope, living cysts show red chlorophyll autofluorescence.

20.4.1.3 *Fibrocapsa japonica* Toriumi and Takano (Fig. 20.5C)

- Cysts of *Fibrocapsa japonica* (Toriumi and Takano, 1973); (Yoshimatsu, 1987, p. 28, Pl. 1, Figs. 2–7).

Shape: spherical, but hemispherical when the cysts adhere to the surface of diatom frustules, and probably covered with thin gelatinous layer. *Size*: small; 15–20 µm in diameter. *Wall relationship and feature*: probably a single layer and transparent without any ornament on the surface. *Opening for germination*: unknown. *Other features*: Living cysts filled with fresh protoplasm are dark orange-brown in colour, and lack a red-pigmented body.

Comments: The cyst of *F. japonica* is similar to cysts of *Chattonella* in being small, transparent, and spherical to hemispherical, but differs from the latter in not having dark brown to black spots.

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Part III

Monitoring and Management

21

Environmental monitoring, with examples from Narragansett Bay

T. J. Smayda

21.1 Introduction

21.1.1 Principles of monitoring

A well-defined objective is essential in designing a HAB environmental-monitoring programme; neither the strategy nor the protocol should be fixed. The programme design varies with the objectives, habitat and HAB populations: What do you wish to monitor? To assess? Why? Are the data to be used for observational purposes, such as tracking blooms? For early warning of potentially harmful species? To document ecological harm or dysfunction? To develop predictive capability? To safeguard aquacultural operations? Selection of the variables and techniques, frequency of measurement, and siting of sampling locations are major considerations. A design that is too superficial may produce data inadequate for their intended use; a design that is too complicated may be needlessly labour-intensive and costly. While investigators should consider the procedures and approaches of studies carried out elsewhere, their own monitoring programme must match their specific objectives and site-specific conditions. The existing database will need to be considered, with decisions influenced by the investigator's experience and intuition. Progressive refinements of the monitoring programme will probably have to be made as experience is gained.

The level of insight required from the monitoring programme, i.e. awareness, observational, quantitative, or predictive, also influences its design. Observational monitoring, i.e. for status and trends, will not require concurrent rate measurements of phytoplankton growth, grazing and nutrient uptake. Monitoring focusing on nutrients and oxygen levels may not require biological measurements. Surveillance for potentially harmful species, i.e. early warning or alert monitoring, can be restricted to seasons when these taxa are expected. Concurrent physical and chemical measurements are probably not needed in that case, unless one hopes to find a physical or chemical variable useful as an indicator, or proxy variable. Proxy variables are often easier and less expensive to monitor, but are inexact predictors of occurrences and blooms of HAB species (Morel and Anderson, 1976), which are under multifactorial control. If proxy variables are sought, statistical correlation with the HAB aspect(s) of interest needs to be evaluated using local observations made over a suitable period. The investigator should avoid use of anecdotal relationships, poorly correlated indices, and local application of proxy correlations established for other regions or species. Monitoring to assess actual or potential anthropogenic

effects on HAB events, such as aquacultural activities or the introduction of novel species through discharge of ballast water (Hallegraeff and Bolch, 1992), requires knowledge of the pre-existing normal (baseline) conditions and their variability, and a long-term monitoring effort to distinguish anthropogenic changes from those attributable to natural variance. Monitoring to develop models for predictive purposes and strategies of pre-bloom mitigation through habitat modification is the most difficult to achieve. State-of-the-art techniques are then required to quantify the numerous rate processes affecting the selection, growth and losses of HAB organisms at appropriate spatial and temporal scales.

21.1.2 Common design elements

Whatever their objectives, monitoring programmes share four common design elements.

- Reliable measurements of the monitored variables are required. The need for quantification is not relaxed if the primary interest is to establish long-term patterns in occurrences and blooms of HAB species, rather than to achieve predictive capability of HAB events; only the scope of the inquiry changes, not the rigour of measurement.
- Site- and organism-specific features influence the monitoring design. Where upwelling is a factor, for example, oceanography must be assessed. At sites where flushing is important to HAB events, this physical feature must be evaluated; where nutrient loading increases, nutrient chemistry should be measured; where aquacultural deployments are made, bottom-water oxygen should be measured. When meroplanktonic species are involved, their resting stage dynamics cannot be ignored, a life-cycle feature irrelevant to sampling designs dealing with holoplanktonic taxa (e.g. *Ceratium*). When motile species are involved, their diel migrations cannot be ignored.
- The design element must deal with common attributes that transcend phylogenetic, organismic and site-specific differences. That is, the monitoring programme must be designed in accordance with the major ecological and behavioural features represented by the HAB taxa, their planktonic life mode, and the habitat factors regulating their occurrence and growth. For example, nutrient availability should not be ignored in assessments of bloom regulation, but it would make little sense to measure silicate in favour of ammonia when non-siliceous phytoflagellates are the focus.
- The functional scales at which HAB organisms respond to their environment and the scales of habitat variability influencing their biology must take monitoring precedence over the convenience scales of investigator perceptions, methods and work ethic (Andrew and Mapstone, 1987). Data collection and interpretation are compromised if the intrinsic variability of life in a fluid medium, or the spatial- and time-dependent patterns and processes characterizing HAB events, are ignored.

The following sections consider some general characteristics of the phytoplankton habitat, life mode, bloom events and variability relevant to the design of monitoring programmes. Some practical guidance in designing HAB environmental-monitoring programmes is also provided, with examples from Narragansett Bay (Smayda, 1998*b*) frequently used to illustrate the points being made.

21.2 Sampling design considerations

21.2.1 Habitat heterogeneity and variability

Continuous physical and chemical changes of variable frequency, duration and intensity characterize planktonic habitats, which are mosaics of multiple, shifting sub-habitats whose physical and chemical properties exhibit sharp spatial and temporal heterogeneity. Daily variations occur in tidal and flushing cycles, current movements, runoff delivery of nutrients and rates of *in situ* utilization and recycling. Seasonal changes occur in temperature, irradiance, precipitation, runoff, nutrient delivery and mixed-layer depth. Long-term changes in physical-chemical and food-web structure also impact on the selection of HAB species. These daily, seasonal and interannual variations in habitat structure are driven by both external (weather) and internal (trophodynamic) events subject to anthropogenic disturbance and modification. Cultural eutrophication, occurring as episodic, acute events or chronically, alters nutrient conditions and 'water quality'. Watershed management practices, such as river-flow modification, deforestation and agriculture, alter runoff, salinity gradients, flushing rates, delivery of nutrients and irradiance. Trophic processes influence nutrient levels and grazing pressure, while fish farming and aquaculture modify water-mass chemical and oxygenation characteristics. Irregular or anomalous HAB responses can accompany unusual physical and climatic conditions (Chang *et al*., 1995). Often stochastic, these events complicate monitoring. Habitat variability and its modification by watershed practices must therefore be considered in designing monitoring programmes, as they can modify and disrupt niches. The opening and closing of niches represent a series of bloom-support and bloom-repression windows which restrict the occurrences and blooms of HAB species to narrow temporal periods of unpredictable occurrence and duration. Given this habitat complexity and multifactoral regulation of HAB dynamics, what measurements should be made during monitoring?

21.2.2 Physical habitat measurements

Among physical factors, temperature and salinity, minimally, should be measured, and at multiple depths along a spatial gradient. Vertical density profiles, if not directly measured, can be calculated from vertical temperature and salinity profiles to evaluate the degree of water-column mixing. This is particularly important in inshore areas subject to riverine discharge, to incursions of offshore current systems, or upwelling. HAB outbreaks often develop after intense rainfall and runoff, followed by a period of intense sunlight. This has led to the paradigm that increased water-column stratification enclosing a patch of chemically modified, surface-layer water provides the bloom trigger. Measurements of the vertical salinity profile can reveal such a condition. If water-mass stratification was already well established, and the HAB outbreak followed a runoff event, a change in the chemical quality of water may be a more significant bloom stimulus than reduced turbulence. Turbulence can diminish population growth in dinoflagellates by inhibiting their cellular division rates (White, 1976) and interfere with phototactic migrations.

From the gradients in horizontal and vertical salinity distributions, the flushing rate can be established, and wind-induced stowing of water in arms or cul-de-sacs of the embayment detected. The ratio of cellular growth rate to flushing rate of a HAB

species influences its population growth rate; blooms are progressively favoured as this ratio increases above 1:1 (Seliger *et al*., 1970, 1971). If advection is too strong, the imbalance between population washout and growth rates may preclude a bloom event. Local meteorological conditions must also be considered during monitoring. Wind-driven stowing of water masses often leads to HAB accumulations in the inner reaches of an embayment (Fig. 21.1), which can be misinterpreted as reflecting particularly favourable growth conditions at those sites rather than a result of wind-induced stowing.

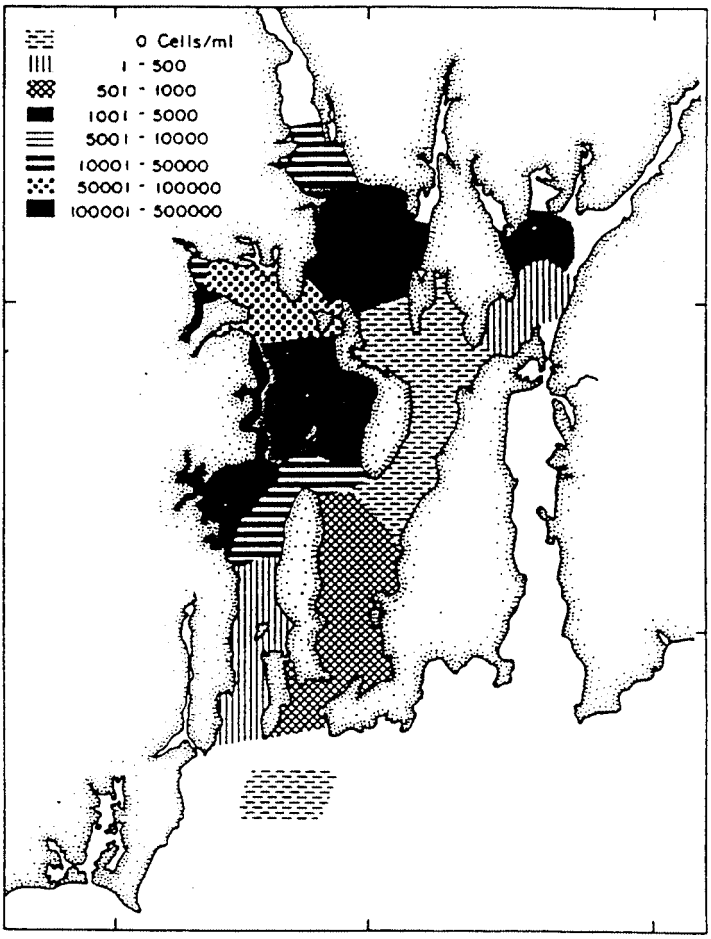

Figure 21.1
Regional abundance patterns of the raphidophyte, *Heterosigma akashiwo*, during a bloom in Narragansett Bay showing the effects of wind-induced stowing that elevated its accumulations in cul-de-sacs and shallow embayments.
Source: Tomas (1980).

21.2.3 Chemical habitat measurements

The chemical habitat, similar to physical conditions, is a mosaic of spatially heterogeneous, temporally variable sub-habitats in which nutrient delivery and concentrations are neither fixed, continuous, nor depend solely on physical and remineralization processes. The chemical milieu is an exceedingly complex mixture of macro-nutrients (nitrogen, phosphorus), micro-nutrients (iron and other trace metals), organic nutrients, 'water quality' factors, and allelopathic secretions by plankton. All have been implicated in affecting HAB processes. Runoff delivery of nutrients and humic substances may increase population-carrying capacity, detoxify the habitat, or in some other way improve overall water quality leading to bloom development in the chemically conditioned water mass (Granéli and Moreira, 1990). The nutrient environment, which is naturally variable and subject to seasonal, regional and long-term modifications, influences HAB events in three major ways: species selection, abundance and bloom dynamics. However, field measurements of habitat chemistry currently provide little reliable, predictive value of whether, where and when a HAB event will occur, or which species will bloom. Measured nutrient levels represent only residual concentrations and provide no information on delivery and uptake rates. Monitoring programmes seeking to establish the latter must include process measurements, along with estimates of nutrient-loading rates based on river flow and content.

The inclusion of macro-nutrient measurements in quantitative and predictive monitoring programmes is strongly encouraged. Long-term measurements of dissolved inorganic NH_4, NO_3 and PO_4 from riverine discharge into recipient waters will reveal trends in macro-nutrient accretion associated with watershed activities and also whether nutrient-enrichment of the recipient waters is occurring. Nutrient measurements prior to, and during, aquacultural activities can establish the nutrient retention and bloom characteristics of the aquacultural site subject to increased nutrient excretion. Chemical conditioning accompanying chronic or episodic eutrophication events may select for HAB species through changes in nutrient ratios that alter inter-specific nutrient resource competition (Smayda, 1989, 1990). Increasing evidence suggests that HAB events in nearshore and more open habitats tend to occur with greater frequency and magnitude when nutrient-enriched and may increase progressively with nutrification (Fig. 21.2) (Smayda, 1989, 1990; Hickel *et al*., 1993; Honjo, 1993). The long-term increases in blooms of *Phaeocystis pouchetii* in Netherlands coastal waters (Cadée and Hegeman, 1986), *Prorocentrum cordatum* in the Black Sea (Smayda, 1990) and flagellates in the German Bight (Hickel *et al*., 1993) are examples of this phenomenon.

Inorganic nitrogen, phosphorus and oxygen are the minimal chemical measurements that should be made, particularly where anthropogenic nutrient modification is occurring. As growing evidence suggests that dissolved organic nitrogen (DON) may be of greater nutritional value to some HAB organisms than expected (Berg *et al*., 1997), measurements of organic sources of nutrient should be considered. Micro-nutrients unquestionably also influence HAB taxa, with evidence strongest for iron regulation and cupric ion sensitivity (see Takahashi and Fukazawa, 1982; Doucette and Harrison, 1990; Anderson and Morel, 1978). However, the stringent sampling (clean techniques) and analytical requirements make routine monitoring of micro-nutrients difficult. Monitoring for 'water-quality' factors (Kondo *et al*., 1990) would appear to be premature, given the rudimentary state of current knowledge and

methodology applicable to this poorly understood aspect of the chemical habitat. Many HAB species supplement their nutrient needs mixotrophically (Hansen, 1998), which further complicates monitoring of the chemical habitat. It is recommended that the investigator concentrate on macro-nutrient monitoring, at least during the initial stages of the programme. Failure to include nutrient measurements will not only compromise the effort, but could result in a loss of information vital to understanding the unresolved links between the chemical environment and HAB events.

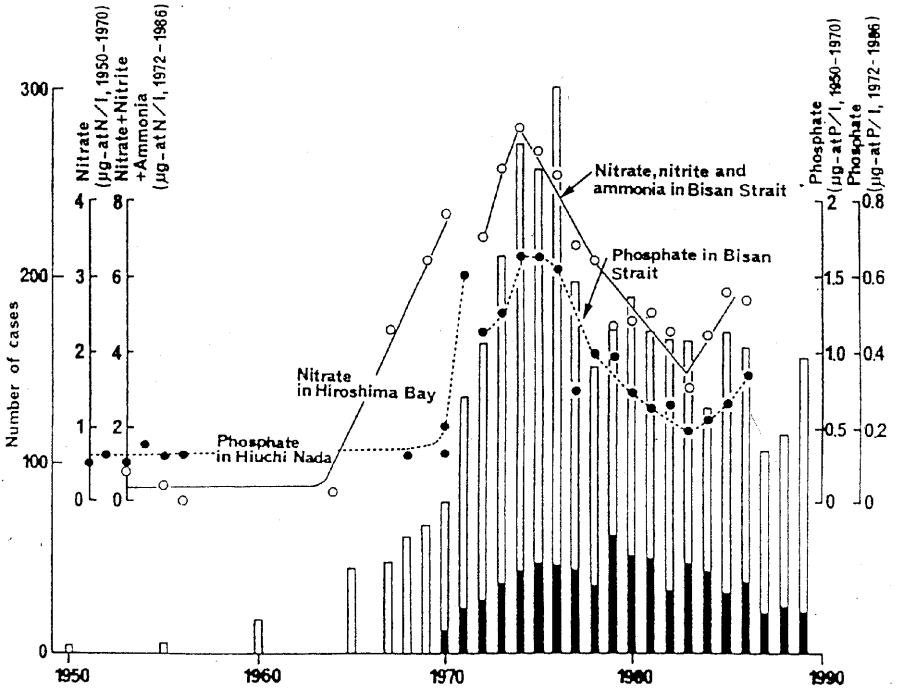

Figure 21.2
Concentrations of inorganic nitrogen and phosphate from 1950–89 in the Seto Inland Sea, the number of cases of red-tide outbreaks (open histograms), and blooms of the raphidophyte, *Heterosigma akashiwo*. *Source:* Honjo (1993).

21.2.4 Oxygen monitoring; control sites

Monitoring of seasonal water-column oxygen levels, with emphasis on bottom-water concentrations, is essential in shallower, poorly flushed coastal waters, at aquacultural sites and in regions exhibiting environmental degradation. Routine monitoring in these regions may be needed if seasonal oxygen concentrations prior to HAB outbreaks decline to low levels and thereafter are vulnerable to hypoxia or anoxia during deposition of ungrazed HAB cells. A progressive decrease in bottom-water oxygen levels can induce dieoffs in poorly flushed regions subjected to increased nutrient loading and phytoplankton abundance. Increased phytoplankton

biomass during poorly grazed bloom events, such as *Ceratium* blooms (Falkowski *et al*., 1980; Hickel *et al*., 1989), became nutrient-limited, sank to bottom-waters, and decomposed leading to hypoxia or anoxia. In some instances, monitoring of chemical oxygen demand (COD) or biological oxygen demand (BOD) might be warranted, an approach that was helpful in designing criteria to curb HAB outbreaks in the Seto Inland Sea (Okaichi, 1989).

If chemical or other environmental perturbations from agro-industrial-domestic waste discharge, sea-floor mining, aquacultural deployments, mangrove destruction, or other habitat modifications are suspected of stimulating HAB events, control sites free from such contamination must be sampled. Without controls, suspected effects of habitat modifications will not be distinguishable from stochastic processes, natural variance or localized events operative within the sampling grid (Andrew and Mapstone, 1987). Controls may be of two forms: a specific, control sampling site, or use of a comparative temporal (i.e. time series) analysis using the data set for the sampling site within the perturbed region. In the latter instance, the retrospective time series must have begun prior to initiation of the environmental modification.

21.2.5 Remote sensing, data buoys, ships of opportunity

Pelagic habitats are open systems subject to incursions of water masses from offshore. Dinoflagellates can traverse significant distances within relatively short timescales during their growth and entrainment within coastal currents, and provide 'seed stock' triggering local blooms during offshore-onshore, alongshore, and 'within-system' dispersions (Figs. 21.3, 21.4; Lindahl, 1986; Franks *et al*., 1989; Franks and Anderson, 1992; Tyler and Seliger, 1978; Tester *et al*., 1991). Seedings leading to blooms have also been associated with upwelling relaxations (Blasco, 1977; Fraga *et al*., 1989; Pitcher and Boyd, 1996), tidal fronts (Pingree *et al*., 1975) and frontal-zone dynamics (Seliger *et al*., 1981; Le Fèvre, 1986). Monitoring programmes must take such dynamics into account, particularly when seeking to protect aquacultural operations, or where concerned with seafood safety.

Monitoring large-scale physically driven events is considerably more difficult than 'standard' monitoring programmes. It requires a large interdisciplinary research team, access to a wide variety of physical oceanographic instrumentation and ships, and is costly. The investigator should consider the use of satellites and other remote-sensing detectors to establish the local incursions, frontal structure and regional movements of such water masses based on their temperature and chlorophyll signatures. Such approaches have been successfully used in open coastal waters (Dundas *et al*., 1989; Kahru *et al*., 1994; Keafer and Anderson, 1993). The use of flow-through systems deployed on ferries crossing standard routes, or other ships of opportunity, to monitor HAB populations and accompanying physical and chemical conditions is another option (Harashima *et al*., 1997; Kononen and Leppänen, 1997). The automatic water samplers used to collect samples for HAB species identification and quantification can be supplemented with sensors for direct measurement of physical and chemical factors and photosynthetic pigments at high frequency and extended duration, at relatively low cost. These surveys can be combined with satellite remote-sensing efforts. Restriction of flow-through sampling to near-surface depths is a handicap. Another option is to use marine data buoys deployed in series and designed for real-time, *in situ* monitoring of plankton, physical, chemical and meteorological variables. The Seawatch Buoy System (Fig. 21.4) deployed along the

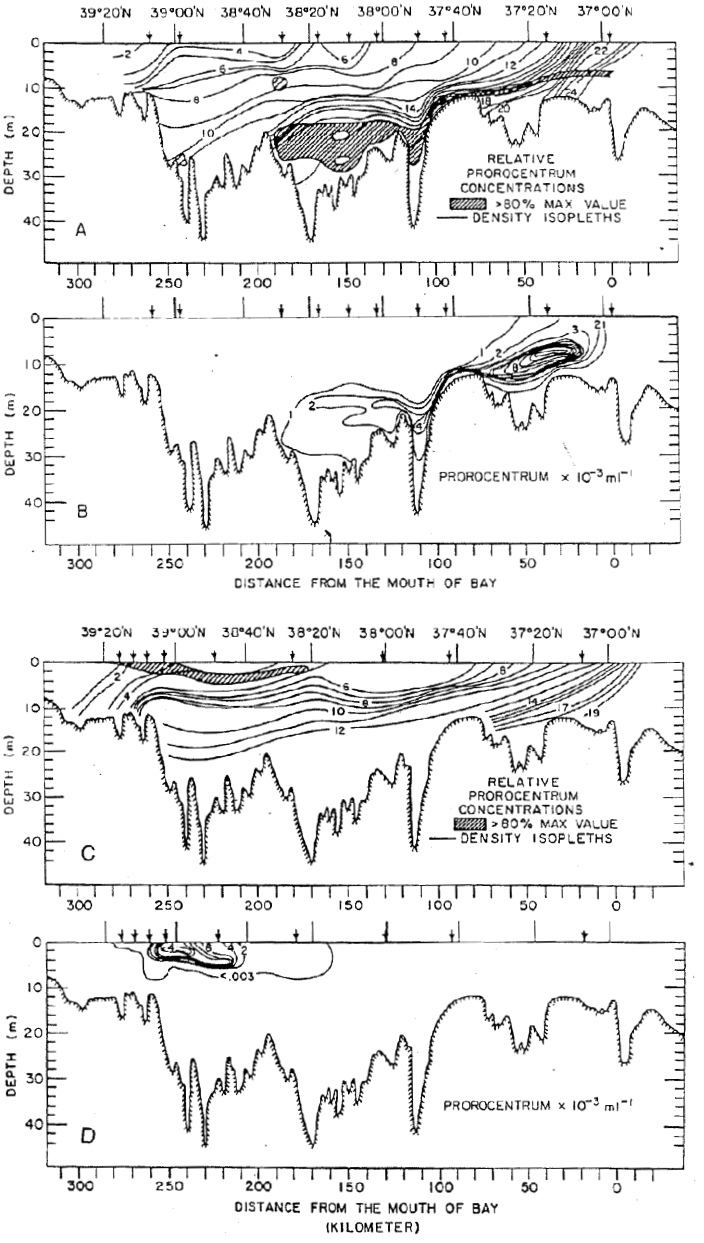

Figure 21.3
Subsurface distribution ($\times 10^{-3}$ cells ml^{-1}) of *Prorocentrum minimum* and its transport within Chesapeake Bay, together with associated density isopleths ($\sigma t \times 10^3$) during late winter (panels A, B) and summer (panels C, D).
Source: Tyler and Seliger (1978).

Norwegian coast and consisting of eight buoys, 40 fish-farm observer sites and 21 mussel-toxicity test sites detects potentially toxic species through a combination of bio-optical, direct counting and toxicity testing (Johnsen *et al*., 1997; Johnsen and Sakshaug, 2000). Forecasting of upstream blooms is facilitated by buoy deployment sites positioned to provide an early warning to the aquaculture industry. Innovative cost sharing finances this elaborate monitoring network, which provides an excellent model for applications in other regions.

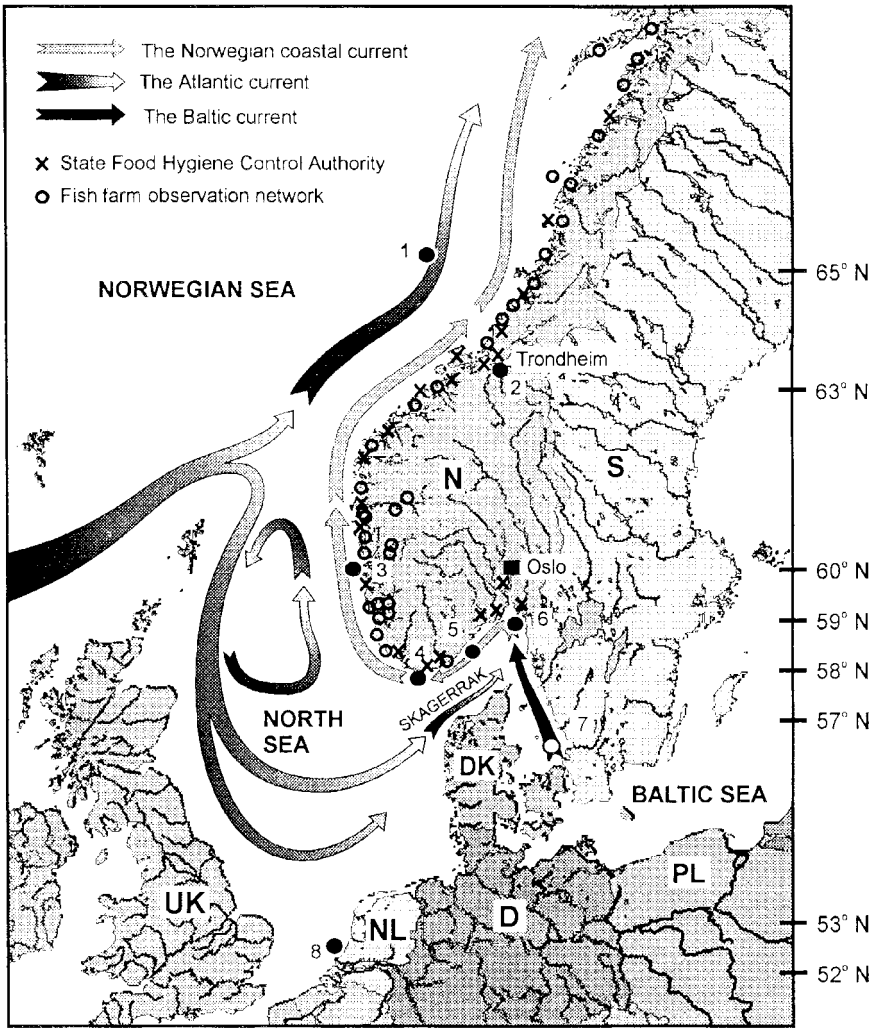

Figure 21.4
The Norwegian coastline showing the location of Seawatch buoy stations, the State Food Hygiene Control Authority sites and fish-farm observation network sites, together with surface-current circulation pattern.
Source: Johnsen and Sakshaug (2000).

21.3 HAB ORGANISMS

21.3.1 Bloom features relevant to monitoring

HAB species must overcome four basic impediments to bloom: a temperature threshold, chemical restraints, interspecific competition and grazing losses (Smayda, 1998*a*). Once started, blooms progress through four phases: initiation, accumulation, stationary and termination, but can abort, regress or accelerate at any stage, for various reasons (Fig. 21.5). Bloom inception and termination may be precipitous or drawn out. Accumulations that result in 'red-tide displays' may be the combined result of logarithmic growth and physical aggregation. During the stationary phase, growth (accumulation) and loss rates are usually in balance. Blooms terminate because of the combined effects of nutrient-limitation, microbial or viral infestation, grazing and advective losses (one of which is usually the primary cause), factors that also influence bloom duration and magnitude. Termination of a HAB event may be followed by blooms of other HAB species, as in Narragansett Bay (Fig. 21.6). Following blooms, autochthonous species may persist at detectable levels of abundance, or remain within the pelagic community as 'hidden flora' undetectable by standard sampling or enumeration techniques, or undergo a life-cycle transformation into their benthic resting stage. Allochthonous species do not survive post-bloom habitat conditions, and must be reseeded to bloom again. Some reseedings will be

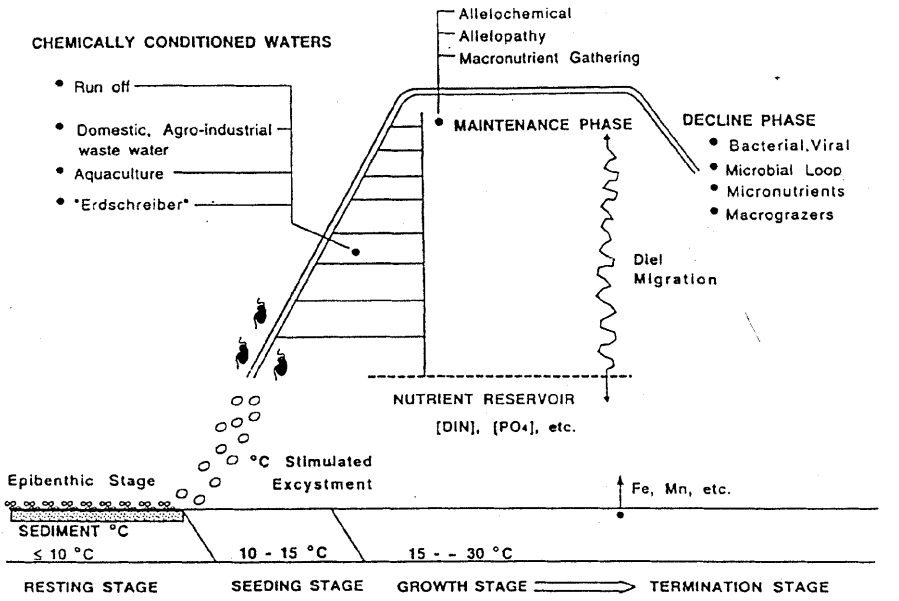

Figure 21.5
General model of key factors influencing initiation, continuance, termination and pattern of HAB blooms, with specific notations given for *Heterosigma akashiwo.*
Source: Smayda (1998*a*).

sterile because the recipient water mass cannot support either survival or blooming of the inoculated species. Novel species seeded anthropogenically must first establish local populations able to compete successfully with the indigenous flora. Multiple introductions may be required before a novel species achieves the foothold needed to allow year-round survival and eventually bloom. This sequence of successful expatriation may take many years. Whether autochthonous, allochthonous or newly invasive species are involved, the variable ability of habitats to satisfy the exacting eco-physiological and bloom-stage requirements of HAB species (Fig. 21.5) contributes to highly unpredictable bloom occurrences.

21.3.2 Species variability and monitoring

The presence of a HAB species does not mean that it will bloom, nor does its absence necessarily rule out that it will not appear at some later date. The regulatory factors that trigger and support blooms differ from those favouring survival and

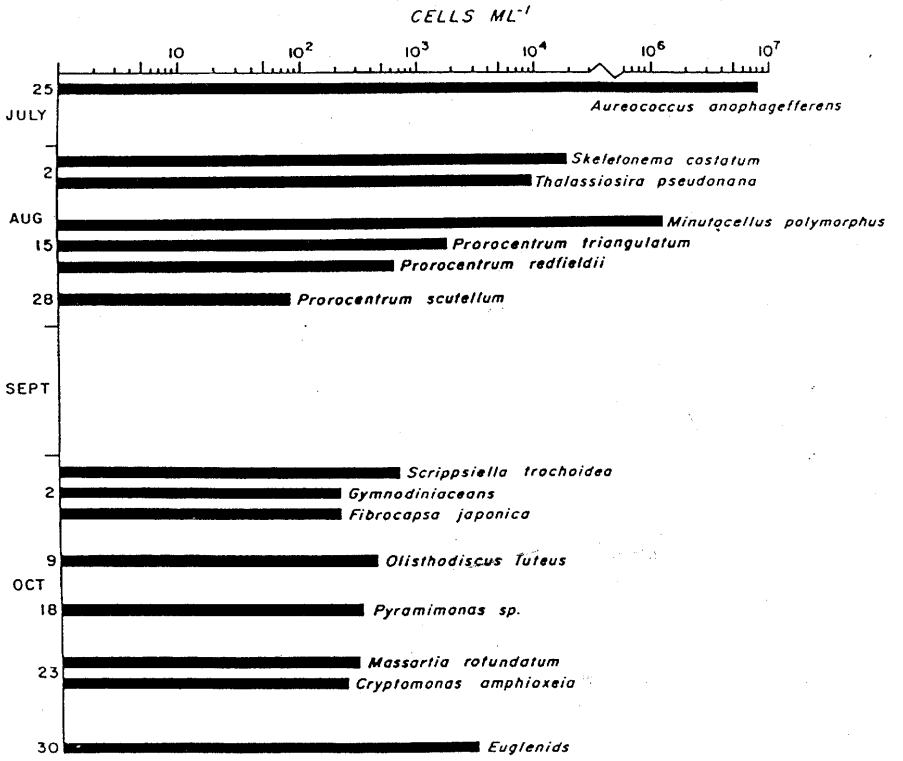

Figure 21.6
Successional patterns of the major bloom species in Narragansett Bay during a 'brown-tide' event, including the time and magnitude of their maximal abundance. Note: 15 different taxa bloomed during this five-month HAB event.
Source: Smayda and Villareal (1989).

availability of seed stock (Fig. 21.5). This aspect and the significant variability that characterizes phytoplankton behaviour complicate the design of HAB monitoring programmes. Variability occurs in various patterns: as cycles, trends, fluctuations, unusual events, irregular pulses; at various scales: daily, seasonally, annually, decadally, etc.; and at various frequencies (Smayda, 1998*b*). Some examples of the interannual and seasonal variability in weekly occurrence and abundance patterns of four bloom species over a 22-year period in Narragansett Bay are shown in Fig. 21.7. *Heterocapsa rotundata* achieved its annual maximum during all seasons. Common from 1959 to 1962, it occurred infrequently in the following 12 years then became abundant again after 1974 and reached its greatest abundance during 1978–1980. *Prorocentrum triestinum* and *P. minimum* (= *cordatum*) exhibited similar occurrence patterns, but the duration of their annual occurrences, abundance and time of summer maximum varied interannually. *Prorocentrum triestinum* exhibited maximal abundances in the mid-1960s; *P. minimum* in the late 1970s. *Heterosigma akashiwo*'s annual maximum usually occurred between weeks 22 and 28, whereas its annual occurrence and abundance patterns varied considerably between years.

There are at least 12 distinct species-specific patterns of variability (Table 21.1), but the behaviour ascribed to the example-species may not apply throughout its distributional range. *Aureococcus anophagefferens*, for example, has become a perennial bloom-species in Long Island coastal embayments following its novel 1985 bloom, but not in Narragansett Bay (Bricelj and Lonsdale, 1997; Smayda, unpublished). *Prorocentrum minimum (= cordatum)* has become more abundant in the Black Sea and Narragansett Bay, but only in the Black Sea has it also become a keystone species (Smayda, 1990). Novel, local appearances may or may not be followed by regional spreading. *Karenia* (*Gymnodinium) mikimotoi* has spread, persisted and bloomed throughout the North Sea since its first registered bloom off the Norwegian coast in 1966 (Braarud and Heimdal, 1970; Partensky and Sournia, 1986), whereas harmful *Chrysochromulina polylepis* has not bloomed with similar regularity and intensity since its novel outbreak in 1988 (Edvardsen and Paasche, 1998).

TABLE 21.1 Some species-specific patterns of phytoplankton variability

Episodic bloom occurrences	*Karenia brevis*
Cyclical bloom abundance	*Thalassiosira nordenskioeldii*
Seasonal shift in predominance	*Skeletonema costatum*
Trend: decreasing abundance	*Heterosigma akashiwo*
Trend: disappearance	*Skeletonema costatum*
Trend: increasing abundance	*Prorocentrum minimum*
Stochastic bloom occurrences	Many taxa
Novel appearances, followed by	
a. persistence	*Aureoumbra lagunensis*
b. perennial predominance	*Aureococcus anophagefferens*
c. establishment as keystone species	*Phaeocystis pouchetii*
d. regional spreading and persistence	*Karenia mikimotoi*
e. aperiodic blooms	*Pfiesteria piscicida*
f. rarity or disappearance	*Chrysochromulina polylepis*

Source: modified from Smayda (1998*b*).

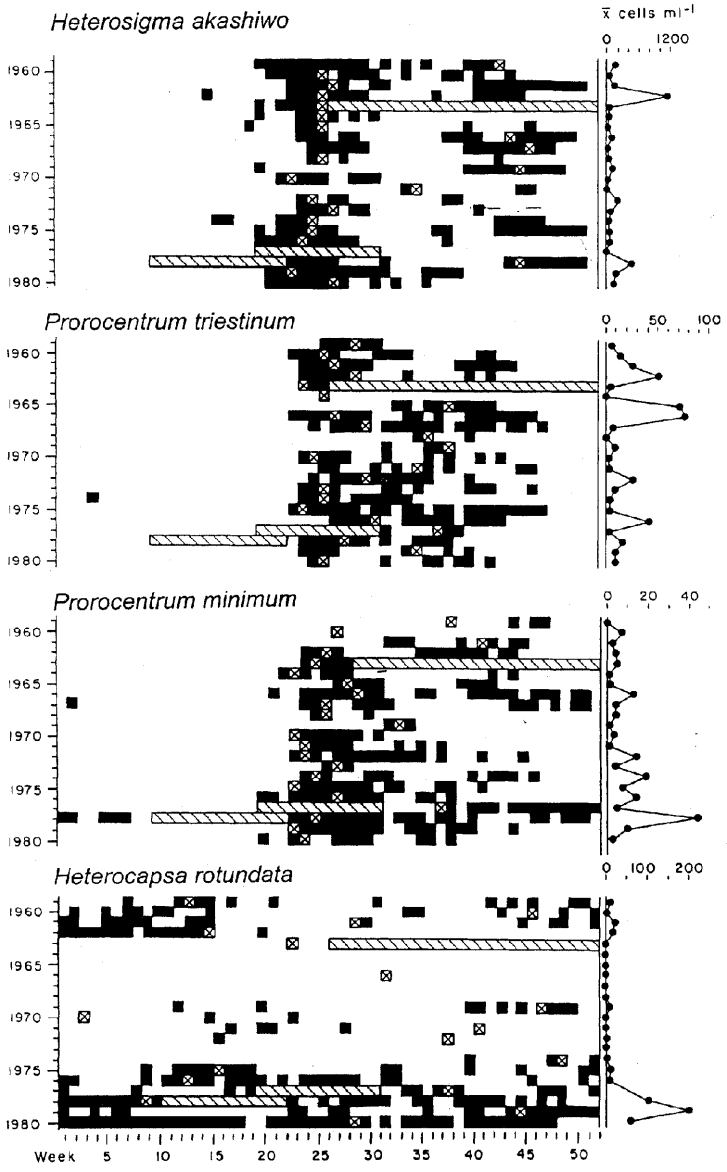

Figure 21.7
Occurrence and abundance patterns of *Heterosigma akashiwo*, *Prorocentrum triestinum*, *Prorocentrum minimum* and *Heterocapsa rotundata* in Narragansett Bay from 1959–1980. X designates the week of maximum abundance during that year. Hatched areas designate periods when samples were not collected. Annual mean abundances are plotted along right margin.
Source: Karentz and Smayda (1984).

21.3.3 Special HAB species characteristics influencing sampling design

In situ distributions of phytoplankton are neither spatially uniform nor do they exhibit smooth gradients in abundance (Cassie, 1963). Considerable spatial discontinuity occurs at every level of community organization and dynamics: in species occurrences and co-occurrences, abundance, successional stage, community diversity and dynamics. Tests for random spatial distribution of phytoplankton based on analyses of departure from *Poisson* occurrence, which describe the distribution of random, rare events (Venrick, 1978*b*), have proved to be negative (Cassie, 1963). Non-random distributions (over-dispersion*)* characterize phytoplankton as a consequence of spatial heterogeneity (patchiness) in growth variables, community processes and physical effects. Designation of this phenomenon as over-dispersion does not refer to the dispersal or physical spreading of phytoplankton within their habitat. Rather, it is a mathematical term that refers to the spread of numerical abundance estimates about the mean abundance estimate for samples collected within a given sampling grid over time. The water discolorations that sometimes accompany HAB outbreaks vividly demonstrate over-dispersions. Even if water discoloration is not evident, contagion, aggregation or clumping of the cellular distributions contributing to non-randomness still occur. Two major factors that contribute to over-dispersion and need to be considered in designing monitoring programmes are micro-distributions and phototactic migrations of HAB species.

21.3.3.1 *Micro-distributions*

Phytoplankton exhibit small-scale micro-distributions partly in response to micro-gradients in growth-promoting conditions. Aggregation of HAB cells into ephemeral bands, streaks and patches of variable size, resulting from wind-induced filaments of circulating water, further complicates sampling (Barstow, 1983; Ryther, 1955). Chapter 2 should be consulted for further discussion of Langmuir Circulation effects. Table 21.2 shows the surface temperature, salinity and accompanying abundance and distributions of three dinoflagellate species sampled at four intervals along 250 m transects at two locations. Along the transect (#1) of nearly uniform temperature, but with a *c.* 3‰ range in salinity, abundance of the individual species varied 5-fold to 10-fold. Along the transect (#5) of nearly uniform temperature and salinity, the abundance of *Ceratium furca* and *Dinophysis acuta* was similar, but varied 3-fold for the more abundant *Prorocentrum micans.* If the monitoring decision was to sample the 5 m depth and not at the surface (Table 21.2B), a significantly lower population of *P. micans* would have been encountered at some of the sampling sites. If only one sample was collected along transect #1, and specifically at 5 m depth at site 1d, the population estimate (540 cells l^{-1}) for P. *micans* would have been *c.* 60-fold lower than that (33,100 cells l^{-1}), had the sample been collected at the surface only 25 m away (site 1c).

21.3.3.2 *Phototactic migrations*

HAB flagellates can exhibit significant diel variations in their vertical distribution because of phototactic behaviour, or semi-diurnal entrainment in internal tides (Kamykowski, 1976), movements which also influence their retention within an estuary (Anderson and Stolzenbach, 1985). Some species exhibit a 'night rise/day descent'; others, the opposite diel pattern (Hasle, 1950, 1954*a*; Blasco, 1978; Kamykowski, 1981). Diel abundance at a given depth in the water column and

among species can vary significantly because of different motility speeds and diel response patterns, as shown in Table 21.2B. Abundance of *P. micans* was fairly uniform in the upper 10 m at transect site 1a, but highly aggregated at the surface at sites 1c and 1d. A consequence of vertical migratory behaviour is that no two samples are strictly comparable, unless they represent the entire water column (i.e. pooled samples) or are collected at the same time of day under the same light conditions. The significant sampling problems posed by diel migratory behaviour have been traditionally neglected in field studies, partly because diel sampling is highly labour-intensive. However, if an objective of the monitoring programme is to develop models for prediction and mitigation, then knowledge of diel migratory behaviour is essential (Kishi and Ikeda, 1989).

Daily variations in vertical distribution patterns can also accompany internal wave dynamics (Kamykowski, 1976) and directed motility along pycnoclines (see Pingree *et al*., 1975) also contributes to over-dispersion. Cassie (1963) concluded that the over-dispersion characterizing phytoplankton precludes application of the ideal sampling strategy, i.e. the collection of only one, or relatively few, samples to estimate accurately population composition, abundance and dynamics. Cassie suggests that more accurate estimations require collection of a large number of

TABLE 21.2 A, small-scale and meso-scale horizontal patchiness at surface; B, vertical patchiness in distribution of dinoflagellates (as cells per 50 ml^{-1}) in Oslofjord. Distance between a and b, c and d, 25 m; between b and c, 250 m; between Stations 1 and 5, 20 km

A. Small-scale and meso-scale horizontal patchiness				
Station	**1a**	**1b**	**1c**	**1d**
Temperature	17.7	17.5	7.5	17.9
Salinity (‰)	11.67	12.74	13.37	14.42
Ceratium furca	6	45	18	31
Dinophysis acuta	2	8	4	10
Prorocentrum micans	148	441	1655	1240
P. micans at 5 m	153	218	149	27
Station	**5a**	**5b**	**5c**	**5d**
Temperature	18.1	17.9	17.9	17.9
Salinity (‰)	16.44	16.20	16.62	16.38
Ceratium furca	107	131	96	80
Dinophysis acuta	82	66	56	75
Prorocentrum micans	450	1170	1386	922
B. Vertical patchiness				
Depth (m)	0	5	10	25
Prorocentrum micans				
Station 1a	148	153	110	2
1b	441	218	16	0
1c	1655	149	19	0
1d	1240	27	14	0

Source: modified from Hasle (1954*b*).

smaller samples (e.g. 1 l samples), rather than a small number of samples of larger volume (e.g. 5 l samples), as well as sampling at a greater number of stations and depths. An integrated sample for the water column or mixed layer composited by pooling the samples collected at the discrete sampling depths can reduce this effort.

21.3.4 Design of HAB species-monitoring procedures

The foregoing attributes of bloom species behaviour, variability and regulation challenge the design of HAB monitoring programmes. HAB bloom events often appear to be stochastic – the result of the bloom species 'being in the right place at the right time' (Smayda and Reynolds, 2001); i.e. being seeded at a time and place when their ecophysiological requirements are accommodated by the bloom habitat. This unpredictability challenges the design of monitoring programmes. For poorly studied regions, it is recommended that the HAB species most likely to occur locally be evaluated first by consulting regional phytoplankton checklists and studies, and then by seeking information on their seasonal occurrence patterns and ecophysiological characteristics. Useful information will be found in available proceedings of HAB symposia. This background information will help to define the periods of the potential appearance of the anticipated HAB species and accompanying physical and chemical habitat characteristics.

While this presence/absence technique is a relatively easy and appropriate first step in monitoring an unknown habitat, it is qualitative, with a considerable loss of information needed to quantify HAB events and dynamics. This type of monitoring will neither explain the absence of an expected species, nor, if present, why it bloomed. Similarly, the initiation of a monitoring programme after a bloom has begun will not be very helpful in diagnosing bloom triggers. The habitat and bloom-cycle features regulating HAB species selection and dynamics (Fig. 21.5) occur at variable rates and combinations that can be quantified only by experimentation. The investigator must guard against the tendency to focus on the HAB species of interest and to ignore other members of the community then present, particularly when tracking the dynamics of bloom species. The super-abundance of the HAB species tends to deflect investigators from considering the co-occurring species and their abundances. The impression usually given in the numerous reports of HAB blooms is that they are *mono-specific* events, but where detailed community analyses have been carried out (see Smayda and Villareal, 1989), this is scarcely the case (Fig. 21.6). The investigator must recognize that the entire community may fluctuate (i.e. respond) as a unit, or as individual species subject to the bloom impediments, patterns and variability considered above (Figs. 21.5, 21.7). In monitoring for a given species, whether as an early warning system or to follow its bloom dynamics, the principal organizational feature of phytoplankton communities, which poses methodological problems, must be dealt with; i.e. a phytoplankton community is a polymixture of species having different abundances; each species may be in a different phase of its own bloom cycle; and collectively these species are transitional components in the progression to the next successional stage. Therefore, once the investigator has gained insight into HAB species occurrence and bloom patterns, quantitative estimates of abundance will become the more desirable index, particularly if ancillary data on physical and chemical environmental variables are being collected.

To summarize, the common plankton attributes characterizing HAB taxa must be considered in monitoring strategies; i.e. the taxa occur in communities of polymixtures of species, have variable abundance, seasonal preferences and exhibit interannual variations in occurrence, abundance and bloom events. These dynamics may be entrained in rhythmic cycles (White, 1987) or show long-term trends (see Smayda, 1989, 1990). Within a given HAB event, several species may exhibit concurrent blooms, with their bloom stages either in or out of phase with each other. Termination of one HAB event may be followed by another, resulting in a series of HAB outbreaks during a given year, such as those found in Narragansett Bay (Fig. 21.6) and in Tolo Harbour, Hong Kong (Wong and Wu, 1987). Monitoring programmes must deal with such situations.

21.4 Design of a monitoring programme

21.4.1 General aspects

Whether the goal of the HAB monitoring programme is purely descriptive, or is to seek statistical correlations between specific HAB characteristics and habitat variables, the basic *in situ* sampling requirements and constraints are similar. The investigator must first define the target variables, i.e. the HAB species, temperature, nutrient, etc., about which information is sought. Precautions must be taken to ensure that the derived conclusions will indeed be applicable to the HAB species, event, habitat, etc. This requires that representative samples be collected, based on a sampling strategy suitable to the characteristics of the variables to be measured. Following selection of the target variables and identification of their relevant characteristics, the investigator must satisfy three additional requirements: identify the sampling devices needed; select the sampling grid; and set up a sampling schedule (Venrick, 1978*a*).

21.4.2 Selection of sample collection sites and observational periods

There are two options in selecting the sample collection sites: to use randomly selected stations or fixed-station sites. Randomly selected stations might facilitate early warning monitoring programmes to detect potentially problematic species, but the habitat and biotic changes that occur between sampling dates compromise this sampling strategy. Fixed-station locations, which are recommended for more quantitative studies, diminish this problem. The number and locations of stations and the duration and frequency of sampling are highly dependent on programme objectives. If the objective is to determine long-term HAB patterns, monitoring obviously must continue over several years. If monitoring for potential HAB events during a given year is the objective, sampling should be carried out during the HAB season; i.e. the period during which HAB or benign red-tide events have previously occurred at that location, or in contiguous waters. If such information is lacking for a monitoring site located in temperate or boreal waters, the period of water-mass stratification, usually from late spring to early autumn, should be targeted for such monitoring. Unexplored tropical and subtropical regions may have greater year-round HAB potential than other biogeographical regions. Year-round stratification often occurs (sometimes interrupted by localized upwelling or enhanced during seasonal monsoons)

which compromises a priori selection of the 'HAB season'. A close watch on meteorological conditions may help to establish situations when the potential for a HAB event increases, either as a consequence of localized growth or of wind-driven accumulations of noxious species, such as *Trichodesmium* windrows.

21.4.3 Frequency and duration of sampling

HAB events are often fast moving, ephemeral and subject to meteorologically induced disruptions or accumulations. Even though the actual growth kinetics may be slow, physical environmental disturbances may determine the speed with which HAB events appear and disappear. The basic monitoring strategy, therefore, should be one of high sampling frequency, particularly when the objective is to determine the environmental control mechanisms. Ideally, samples should be collected daily, but certainly not less than twice a week during longer-lasting blooms. The logistics of this sampling requirement obviously are more easily met in coastal regions, from which land-based monitoring operations can be launched, than in continental-shelf waters and inland seas requiring expensive ship-based operations and large spatial sampling grids (see Sections 21.2.5 and 21.6.1). Diel sampling may sometimes be needed to unravel the basic features of the phototactic, migratory behaviour of HAB flagellates (Fig. 21.8) as a factor in the bloom episode (Blasco, 1978; Hasle, 1950, 1954*a*; Kamykowski, 1981). In early warning programmes, frequency of sampling might be sacrificed for sampling an increased number of stations to achieve greater regional coverage. When it is not possible to sample all stations on a given date because of the size of the sampling grid and workload, the remaining stations should be sampled as soon as possible thereafter to typify more accurately HAB dynamics during that phase of its bloom cycle.

The duration of monitoring is not fixed; it must vary with the HAB event. HAB events lasting less than a week are not unusual, but events have lasted for five months in Narragansett Bay (Smayda and Villareal, 1989) and even for years, as in the case of a brown tide in Laguna Madre, Texas (Stockwell *et al*., 1993). The objectives of the programme will also influence sampling duration.

21.4.4 Population analyses

By definition, a bloom occurs when a species has increased in abundance. If the bloom event is first signalled by increased toxin levels in shellfish, or dieoffs of fish and other biotic groups, the investigator's first objective is to identify the causative organism(s). Reliable taxonomic identifications are essential in population analyses. If the bloom species cannot be determined for some reason, suitably preserved samples should be archived for future identification by the investigator or for submission to taxonomic experts. If a monitoring programme is already on-line and a toxic species appears, its population abundance must then be closely monitored to track a potential harmful bloom outbreak. The frequency of sampling should then be increased, with re-evaluation of whether the number of collection sites routinely being sampled is adequate.

Historically, phytoplankton abundance has been measured primarily as cell numbers based on microsocopic enumeration, or as total community chlorophyll biomass. Microscopic identification and enumeration of phytoplankton species can be tedious, time-consuming and fatiguing, but are indispensable to population

analyses. Advantages include the fact that individual cells are examined, thus allowing evaluation of their cellular condition; the detection of cell-cycle stages, attack by parasites and other microbial agents; and insights gained into bloom-cycle dynamics and species successions. While *cell number* is the preferred measure of HAB population abundance, use of numerical abundance as a measure of standing stock is sometimes compromised by the considerable interspecific differences in cell size characteristic of phytoplankton species generally (see Chapter 4). Numerical estimates of abundance tend to overestimate the contribution of small cells and underestimate that of large cells. Where this may be a problem, the investigator should consider also determining the cellular volumes of the problem species and tracking their abundance and population dynamics expressed as both cell numbers and equivalent cell volume.

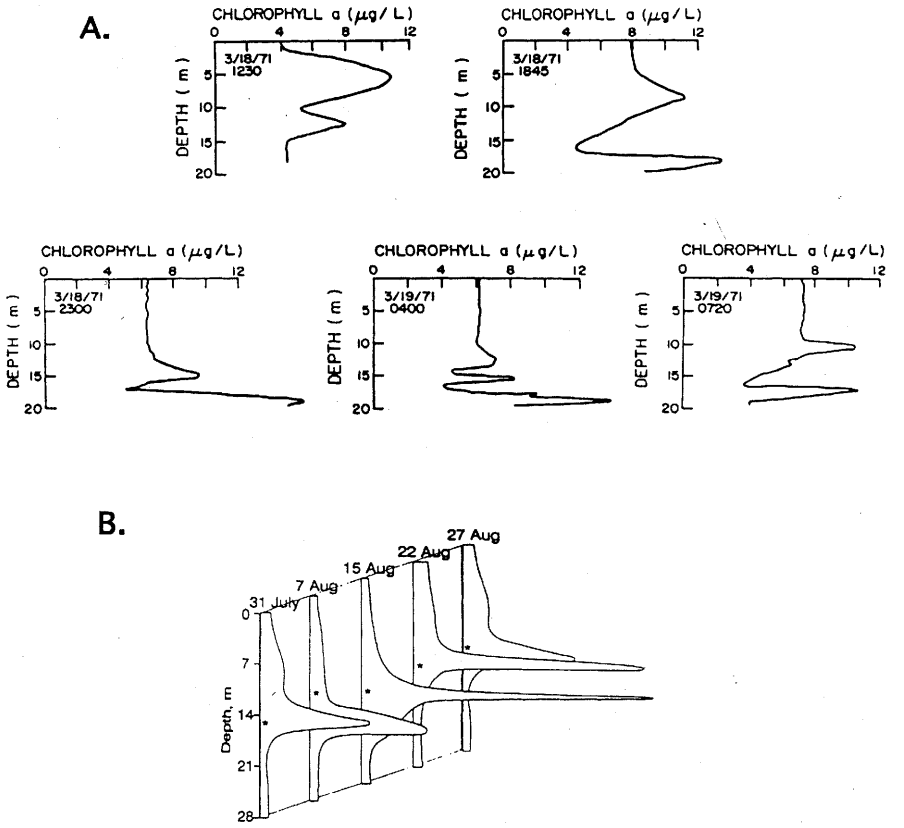

Figure 21.8
Examples of dinoflagellate *in situ* depth-keeping and aggregation into thin-layer strata within the water column. A, diel behaviour of *Akashiwo (= Gymnodinium) sanguinea* (Lasker and Zweifel, 1978); B, persistent aggregation of *Karenia* (= *Gymnodinium) mikimotoi* into thin layers at a North Sea location (Bjørnsen and Nielsen, 1991).

Chlorophyll measurements are not adequate indicators of population levels or bloom dynamics of HAB species, whose impacts are highly species-specific and whose abundance levels are difficult to retrieve from total community chlorophyll measurements. (Measurement of specific pigments unique to the HAB species for potential use as a quicker, reliable measure of abundance requires technique development, see Jeffrey *et al*., 1997). Even in virtually mono-specific blooms, chlorophyll levels may be an inadequate and incomplete measure of abundance. For example, chlorophyll levels during a five-month harmful brown-tide outbreak of the 2 µm non-motile picoplankter, *Aureococcus anophagefferens*, in Narragansett Bay were similar to the winter–spring bloom maximum. *Aureococcus* was harmful to some trophic components, not because of its biomass, but because of its exceptional numerical abundance (2×109 cells l^{-1}), reduced light transmission limiting macroalgal photosynthesis and blocking filter-feeding in mussels, leading to a dieoff of both trophic components (Smayda and Villareal, 1989). At the other extreme, diarrhetic shellfish poisoning of humans associated with the dinoflagellate genus *Dinophysis* may occur at very low population densities of the latter, $<10^3$ cells l^{-1} (Belin, 1993).

Thus, inimical effects during a HAB episode may result from very high or very low biomass bloom events, occur at very low or very high cellular abundances and be due to very small, or very large, species. Within this variance, the same HAB species may be simultaneously inimical to different trophic or within-trophic components, and for different reasons. The anecdotal perception of a red-tide bloom or HAB event as being of exceptional cellular abundance which leads to water-mass discoloration is incorrect. If such visual display stimulates the monitoring effort, the investigator must understand that the factors triggering this bloom are no longer accessible. Monitoring initiated upon water-mass discoloration can only describe bloom dynamics and associated environmental conditions beginning at an unknown, and most certainly late, stage in the bloom event. Reliance on a visual population abundance level, or formal definition of what constitutes a harmful algal bloom is unreliable, impractical, and unhelpful in guiding the design and initiation of monitoring HAB events.

In interpreting the associations between the HAB event and other biological and chemical variables monitored, the investigator must recognize that the observed HAB dynamics are influenced by overall community structure and interspecific competition among the phytoplankton species and grazers (Fig. 21.5). The presence of each species, for example, reduces the capacity of the environment to support all other species competing for a common pool of available nutrients. Such interactions cannot be ignored (see Barnes and Hasle, 1957). If observations on herbivorous zooplankton are lacking, conclusions regarding the selection or bloom regulation of HAB species based on statistical correlations using this incomplete database must be tempered.

21.5 Example of the Narragansett Bay monitoring programme

Table 21.3 summarizes a monitoring programme in Narragansett Bay (NarrBay) that may help others in designing their own efforts. Although designed to describe and correlate patterns, trends and variability in phytoplankton dynamics and habitat

conditions, this programme has proved to be suitable for monitoring of HAB events (Karentz and Smayda, 1984, 1989; Smayda and Villareal, 1989; Li and Smayda, 2000). The variables measured and sampling design elements in the NarrBay programme are common to phytoplankton monitoring generally; but the number of stations, selection of sampling depths, etc., will vary with site-specific properties and monitoring objectives. The use of continuous vertical-profiling instruments will also influence the monitoring protocol.

In the NarrBay programme, a permanent station is sampled at weekly intervals (since 1959) at a representative site (Station 7 in Fig. 21.9), which is also the down-bay end member of a seven-station transect (ending at Station 2 in Fig. 21.6) extending some 40 km upbay along a gradient of increasing nutrient and decreasing salinity. Transect stations 1 to 6 are sampled periodically for special studies not resolvable by sampling at the permanent station only. The transect is also reactivated during HAB and benign red-tide events, with the sampling frequency then increased to two or three times weekly. Except for seasonal stratification in its innermost reaches, Narragansett Bay is well-mixed all year round. This feature and its relatively shallow mean depth (9 m) led to the selection of three depths (top, mid, bottom) for routine sample collection. Mid and bottom sampling depths are selected at the time of each sampling from on-station measurements of water-column depth. The mid and bottom collection depths vary with tidal phase; samples are collected without regard to tidal phase. At each depth, a 5 l sample is collected by water bottle, from which a 2 l subsample is taken and further subsampled for nutrient and

TABLE 21.3 Phytoplankton monitoring protocol carried out in Narragansett Bay at a long-term, permanent station (7 in Figure 21.9) and at transect stations sampled periodically during red-tide blooms

Variables measured				
Meteorological	Physical	Nutrients	Phytoplankton	Zooplankton
River runoff	Water (°C)	NO_3	Species composition	Species composition
Precipitation	Salinity	NH_4	Numerical abundance	Numerical abundance
Wind speed	Secchi disc	PO_4	Chlorophyll biomass	Dry-weight biomass
Irradiance		SiO_3	Primary production	C, N, biomass
			Size fractionation*	

Stations: one permanent station; seven transect stations sampled periodically.

Sampling depths: three; samples from these discrete depths also used to prepare a 'pooled' sample for measurements of average water-column conditions; for zooplankton, oblique tows of entire water column are made.

Sampling frequency: weekly at permanent station and (when sampled) at transect stations; during bay-wide red-tide blooms, samples collected two to three times weekly at all stations.

Sample volume: 2 l discrete samples collected for salinity, nutrients, phytoplankton species composition, numerical abundance, chlorophyll.
3 l 'pooled' sample prepared for measurements of primary production or size fractionation analyses, and analyses of nutrients and phytoplankton variables.

*Total, 20–60 μm, <20 μm and <10 μm size-classes fractionated for measurements of species composition, numerical abundance, chlorophyll and primary production.

Source : Smayda (unpublished)

phytoplankton analyses (Table 21.3). A 3 l pooled sample is also composited (when needed) by mixing equal volumes of seawater collected from the three sampling depths. Pooled samples are an alternative option to the use of discrete samples to determine average or integrated water-column levels of phytoplankton abundance, species composition, primary production, and for phytoplankton size-class fractionation (Table 21.3). As the causative species of HAB events usually fall within a

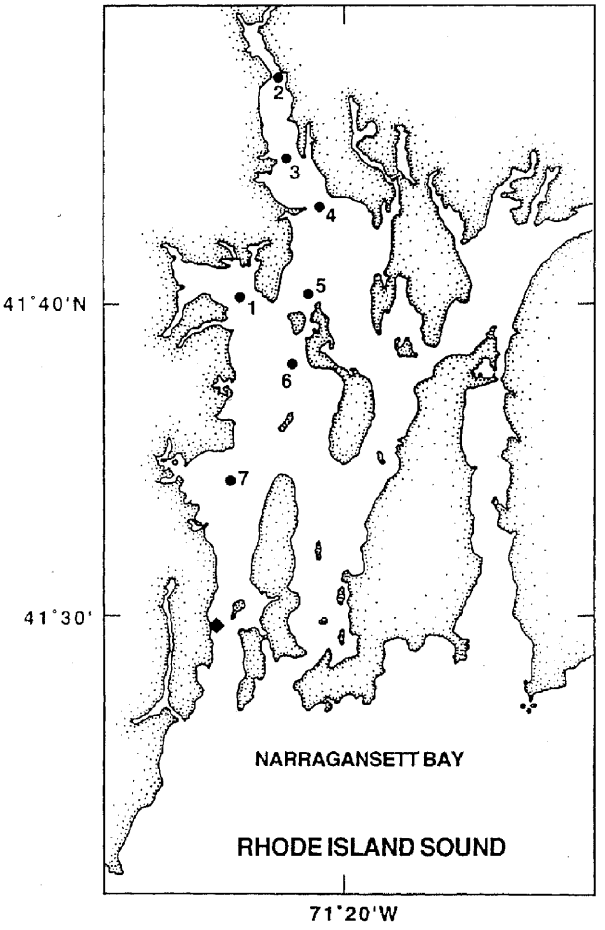

Figure 21.9
Locations of long-term monitoring station (Station 7) in Narragansett Bay and transect stations (1–6) sampled aperiodically for special studies and when red-tide and HAB events occur. Along the gradient from Station 7 to Station 2, mean annual surface salinity decreases from *c*. 31 psu to 20 psu; inorganic nitrogen concentrations increase approximately 10-fold; PO_4 concentrations increase *c*. 5-fold, and SiO_2 concentrations increase *c*. 3.5-fold
Source: Smayda, unpublished data.

narrow size-class band, or are potent at relatively low levels of abundance, i.e. *Dinophysis* spp., size fractionation facilitates assessment, particularly when the desired information cannot be accessed from total community analyses. The pore, or mesh, size used to size fractionate varies with the size of the phytoplankters of interest. Of the variables listed in Table 21.3, only size fractionation is not a routine measurement. For zooplankton collections, oblique tows of 153 µm aperture nets (30.5 cm mouth diameter), fitted with a calibrated flowmeter, are made from bottom to top of the water column while underway. Vertical, top-to-bottom net tows of various mesh sizes are used to collect phytoplankton and microzooplankton for qualitative analyses.

In the NarrBay programme, the time between completion of the sampling and return to the research laboratory is usually several hours. Samples are stored within darkened coolers and transported live for onshore processing, with filtration, freezing, sample preservation and related processing procedures carried out shipboard, where necessary.

Of the variables identified in Table 21.3, a routine monitoring programme should at minimum include measurements of the meteorological, nutrient and physical factors listed; while for phytoplankton, monitoring of species composition, numerical abundance and chlorophyll biomass is essential. Routine measurement of primary production is not essential, unless information on processes and rates is needed. In the NarrBay programme, subsamples from the pooled sample are spiked with ^{14}C and incubated in an outdoor tank at five light levels (100%, 60%, 25%, 10% and 3% of incident irradiance) to establish photosynthesis versus irradiance curves and *in situ* production rates. For zooplankton, measurement of dry-weight biomass based on quantitative collections is essential. Measurements of species composition and numerical abundance will require participation of a zooplankton specialist. A trained phytoplankton specialist can carry out the recommended nutrient measurements, phytoplankton species composition and abundance, and zooplankton dry-weight measurements. In the NarrBay programme, and exclusive of field collection, about two days are required to process samples for each sampling date at the permanent station. Inclusion of primary production measurements and processing collections made at the seven transect stations, however, are labour-intensive requiring about a week and at least two trained personnel to complete the analyses for each sampling date.

Meteorological measurements (Table 21.3) are important in phytoplankton monitoring efforts, as much of the observed dynamics and variability are weather-driven. Such data are usually available at no cost from agencies that routinely monitor and record weather and river flow data. The NarrBay programme measures nutrients by Autoanalyzer, using standard, well-documented procedures. In programmes lacking such instrumentation, the need to use non-automated procedures should not be a deterrent to making these essential measurements. In the NarrBay programme, phytoplankton samples are usually counted live by microscope, using 1 ml Sedgwick-Rafter counting chambers; during size-fractionation analyses, concentrated samples are often enumerated using the techniques described in Durbin *et al*. (1975). For measurements of chlorophyll and other phytoplankton pigments, the handbook edited by Jeffrey *et al*. (1997) should be consulted. For measurement of zooplankton dry-weight biomass in the NarrBay programme, the collections are returned live to the laboratory, split into two equal aliquots, with one aliquot used to determine dry weight, after drying at 60°C, and the carbon and nitrogen content of this biomass determined following

Sharp (1974). The other aliquot is preserved and zooplankton species composition and abundance determined using standard techniques.

An illustration of the value of the long-term NarrBay monitoring programme (i.e. monitoring power potential at other locations) is shown in Fig. 21.10, which gives the mean weekly abundance of the raphidophyte, *Heterosigma akashiwo*, based on weekly sampling from 1959 to 1996 at the long-term monitoring station (Station 7 in Fig. 21.9). Data analysis of this 38-year time series revealed a 94% probability that *Heterosigma* would appear during a given year, with detectable levels *(*1000 cells l^{-1}) rarely occurring before week 20 (Li and Smayda, 2000). There was an 86% probability that its initial, annual appearance would not occur until water-mass temperatures reached a threshold of 10–11°C. Following *Heterosigma*'s appearance, there is then an 80% probability that its bloom maximum will occur sometime between weeks 23 and 26, and a 66% probability that a second bloom would develop between weeks 43 and 46. A monitoring strategy for use by local aquaculturists to ward off potential inimical *Heterosigma* effects on their industry has been developed based on these insights.

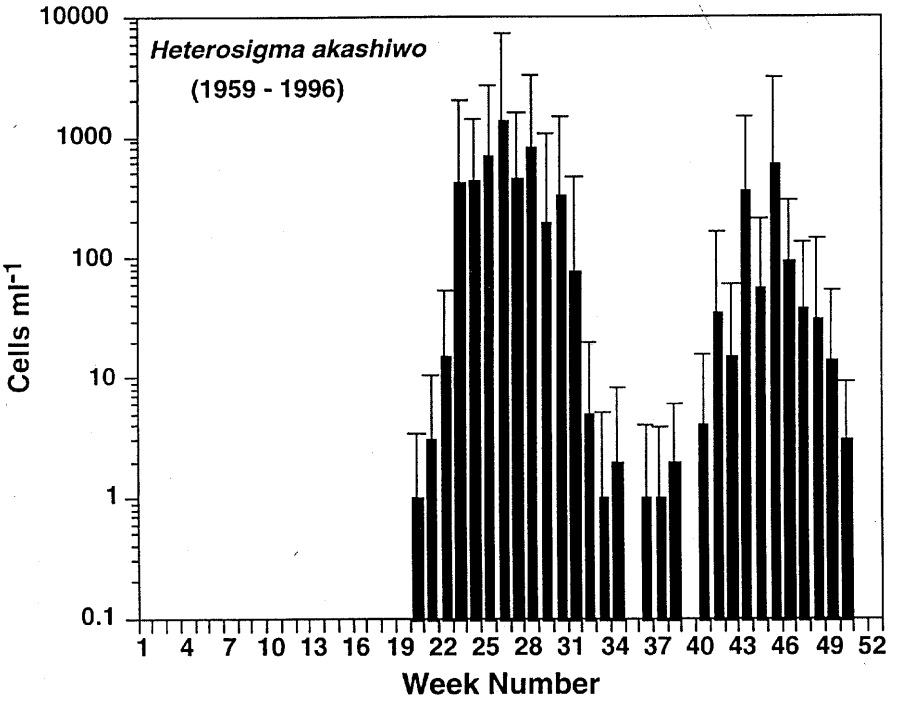

Figure 21.10
Mean weekly surface abundance of *Heterosigma akashiwo* at monitoring Station 7 (Figure 21.9) in lower Narragansett Bay from 1959 to 1996. Range bars above histograms indicate one standard deviation.
Source: Li and Smayda (2000).

21.6 Some specific types of monitoring survey

Several types of monitoring survey are worthy of special comment.

21.6.1 Large-scale regional monitoring

There is an onshore-offshore gradient in the relative and potential importance of physical and chemical parameters in regulating HAB events. In shallower, inshore regions, nutrient dynamics appear to be more significant. In deeper, offshore waters, i.e. along the continental shelf and within inland seas, physical factors such as upwelling events, frontal-zone dynamics and alongshore currents appear to be more significant. Nutrient delivery mechanisms in such physically driven systems differ from those inshore, where riverine influences may predominate. The logistics of monitoring offshore regions and inland seas are more difficult and costly (see Kelley, 1976). Fairly long intervals between surveys will probably be needed, and will compromise sampling at the timescales appropriate for quantifying HAB dynamics in offshore waters.

Numerous reports in the literature indicate that HAB events in inland seas are associated with both nutrient enrichment and physical processes, with a progressive extension of enrichment and HAB outbreaks spreading into offshore water masses, as in the inner Adriatic Sea (Justic *et al*., 1987), Baltic Sea (Kononen, 1992), Black Sea (Bodeanu, 1993), North Sea (Hickel *et al*., 1993; Reid *et al*., 1990) and Seto Inland Sea (Okaichi, 1989). Monitoring in such regions therefore requires a combination of approaches used for systems which are chemically dominated versus physically dominated. Given the large spatial scales and multinational borders that usually characterize inland seas, multi-institutional and multinational collaborative monitoring efforts are desirable. The impressive HAB monitoring and information exchange systems developed for the Seto Inland Sea (Okaichi, 1989) and Norwegian coastal waters (Fig. 21.4; Johnsen *et al*., 1997) illustrate the effectiveness of this approach.

Local HAB events may be under meso-scale meteorological and climatological influence or associated with a regional-scale bloom. For example, a 1985 brown-tide event, which lasted five months in Narragansett Bay, began synchronously over a 500 km stretch of coastline (Smayda and Villareal, 1989). The mechanisms driving large-scale regional HAB events are difficult to quantify, as dealt with in Section 21.2.5. Their detection and explanation will be uncertain aspects of all monitoring programmes, as when local HAB events are part of a meso-scale event the locally monitored behaviour will also reflect local, site-specific regulation. On-line monitoring programmes should be alert to regional-scale meteorologic and hydrographic events, and concurrent reports of bloom events in contiguous waters, particularly if they are unusual or anomalous.

The widespread, unexpected occurrence and spreading of HAB taxa are relevant to regional-scale monitoring programmes, such as the behaviour of *Karenia* (*Gymnodinium*) *mikimotoi* within the North Sea, since its first sighting in 1966 (Dahl and Tangen, 1993; Partensky and Sournia, 1986), the tropical Indo-West Pacific spreading of *Pyrodinium bahamense* var. *compressum* (Hallegraeff and MacLean, 1989), and the dispersal of *Chrysochromulina polylepis* in Scandinavian waters (Granéli *et al*., 1993). Such dramatic large-scale expatriations may be unusual, but must be anticipated. In regions within the potential line of dispersal of the novel

HAB species being spread through current systems, the initiation of monitoring efforts to detect such taxa, if introduced, should be considered, particularly at aquacultural sites. Establishment of a fixed, permanent monitoring station as recommended above and used in the NarrBay programme, would provide such coverage.

21.6.2 Special case of aquacultural site monitoring

Diffuse but growing evidence suggests that the sites of aquacultural deployments eventually may exhibit HAB events influenced somehow by sea farming. In the case of caged culture of finfish (Horner *et al*., 1990), this may simply result from enclosure preventing the penned fish from escaping damage; the HAB event might otherwise have been a normal bloom event in these waters. Early warning monitoring might detect the presence of harmful taxa sufficiently early to allow the temporary removal of fish to safer waters, as done with penned salmon culture enclosures during a *Chrysochromulina polylepis* bloom in Norwegian coastal waters (Underdal *et al*., 1989). Three major environmental modifications associated with the aquaculture of filter-feeding shellfish may stimulate a HAB event and/or lead to dieoff. Filter-feeding shellfish selectively ingest the natural phytoplankton community. This significant predation pressure influences phytoplankton community structure, succession and abundance, subject to further modification, if disease-control agents are applied. Considerable nutrient excretion also accompanies filter-feeding. Decomposition of waste material deposited on to the sediments underlying the culturing rafts, which may be supplemented by ungrazed HAB biomass, reduces oxygen levels. The potential for hypoxic or anoxic conditions leading to dieoffs then becomes of concern. The extent to which these factors may combine to favour HAB outbreaks is influenced by the degree of flushing which varies seasonally and interannually. Increased flushing will contribute to better oxygenation of bottom-waters; wash out slow-growing HAB taxa; replenish the water column with the more natural phytoplankton community associated with that season and location; and dilute HAB growth-promoting factors resulting directly from the aquacultural activity. Hence, aquacultural activities in some locations may result in environmental modifications seemingly favourable to HAB events, creating the need for a product-safety monitoring programme.

21.6.3 Global environmental monitoring and HAB events

Growing international concern over the extent to which the increasing reports of HAB events represent an actual global increase requires a global network of monitoring sites to resolve this important issue. As such a network is currently lacking, the design of the monitoring programme need not conform to an on-line global standard, i.e. an inter-calibrated network. However, the investigator is strongly encouraged to design a programme suitable for retrospective inclusion in a global monitoring network. This effort, which could then be upgraded, if necessary, to meet any future global environmental monitoring design, would require quantitative sampling and measurements to be carried out at appropriate frequency and duration to provide representative information on the monitored habitat. The establishment of at least one permanent, long-term, fixed-station sampling site is therefore strongly encouraged (if a multiple series cannot be managed), independent of the specific local monitoring needs. Measurements at this station should at minimum include temperature, salinity, light transmission (Secchi disc, or other measurement), NH_4, NO_3,

PO_4 and SiO_2, phytoplankton species composition and numerical abundance, chlorophyll and some measure of zooplankton abundance (see Table 21.3). This approach would not only provide a mechanism of long-term surveillance within the sampled habitat. HAB events fall within a class of biological oceanographic problems whose description and solution require interregional, comparative ecological analyses based on detailed site-specific studies. Resolution of whether a global increase in HAB events is occurring and, if so, the nature of its planetary biogeochemical regulation, must be based on the collective patterns revealed by local, regional and subregional monitoring efforts.

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22

Harmful algal monitoring programme and action plan design

P. Andersen, H. Enevoldsen and D. Anderson

22.1 Introduction

The reasons for establishing HAB monitoring are manifold and may include protecting public health, fisheries resources, ecosystem structure and function, and coastal aesthetics (Table 22.1). The appropriate design of a monitoring programme for harmful algae requires an understanding of at least some of the many factors that regulate the dynamics of HABs and the manner in which they cause harm but, by itself, that knowledge does not provide protection of resources. In order to protect resources, application of management and mitigation strategies of many different types is needed. An effective monitoring/management system for HABs therefore must have a variety of elements. As an example, impact prevention requires that the monitoring programme be designed to detect cells or toxins sufficiently early to take appropriate management actions. Those management actions should be clearly defined for each of the many different types of HAB impacts.

Table 22.1 Some typical objectives and goals of HAB and environmental water quality monitoring programmes (modified from Andersen 1996)

Monitoring Goals
HAB monitoring:
• prevent algal toxins from reaching human consumers of shellfish;
• protect humans from algal toxins delivered via sea spray or direct contact (e.g., *Karenia brevis* and *Pfiesteria piscicida* toxins);
• prevent algal toxins from reaching consumers of drinking water (from surface waters or desalination plants);
• minimize damage to living resources such as shellfish and fish;
• minimize economic loss to fishermen, aquaculturists, tourist industry, etc.
General water quality monitoring:
• establish basic knowledge about form and function of the ecosystem investigated and the extent to which it is influenced by anthropogenic factors;
• establish detailed knowledge about selected ecosystem processes to make it possible to understand and predict ecosystem response to eutrophication or exceptional physical and biological events;
• establish patterns and trends for algal populations.

Most of the existing HAB monitoring programmes (87%) are designed by government agencies to fulfil a combination of national, regional and international obligations and objectives (Andersen, 1996). They typically include regulations of shellfisheries in relation to occurrence of algal toxins in shellfish flesh and the occurrence of the algae known to potentially produce the accumulated toxins. Other HAB monitoring programmes may be designed and implemented by private companies to protect aquaculture (6%), or as cooperative monitoring programmes between government agencies and private companies (9%) (IOC, 2001). Resource-specific HAB monitoring programmes exist in many countries/regions, and most HAB monitoring programmes also aim to provide public health safety. Examples of typical objectives of these programmes are given in Table 22.1. Table 22.2 provides an overview of which resources various countries are protecting through establishment of HAB monitoring programmes.

22.2. THE DESIGN ELEMENTS OF HAB MONITORING PROGRAMMES

A HAB monitoring programme is composed of a number of design elements. These must reflect: (i) the objectives of the monitoring programme; (ii) the facilities and resources available; (iii) the specific demands of the end-users of the data; and (iv) the legislation and regulations imposed by the responsible national or regional authorities.

The design of a given HAB monitoring programme must obviously be adapted to local conditions and circumstances and, wherever possible, be interfaced with other monitoring efforts, such as those for general environmental monitoring. It should also take into account the physical and biological regime, available technology, expertise and competence of the staff to carry out the monitoring and management procedure, as well as the local tradition for administration (Andersen, 1996).

However, there are a number of basic or generic elements of a HAB monitoring programme (Figure 22.1):

- Sampling of plankton, shellfish, fish and water
- Analysis of the samples (identification of harmful algae, quantification of harmful algae, measuring toxicity in water, shellfish or fish)
- Environmental observations such as discoloration of the water, fish kills and other animal behaviour
- Evaluation of results
- Dissemination of information and implementation of regulatory action
- Action plans/Mitigation measures.

22.2.1 Acquisition of data

The acquisition of data involves observation, sampling, examination of samples and analysis of raw data. The parameters to be monitored depend on the resource being protected by the monitoring and the associated action plan. Table 22.3 gives an overview of the linkage between the resource to be protected from HAB effects, the potential HAB impact on the relevant resources and the basic parameters to be monitored in order to manage the different resources.

TABLE 22.2 Overview of which resources various countries are protecting through establishment of HAB monitoring programmes. (IOC, 2001)

Country	Fish culture	Fish wild stocks	Mollusc culture	Molluscs wild stocks	Crustacean culture	Natural ecosystems	Public safety	Quality control of products	Recreational/ Tourist aspects
Australia–Tasmania	X		X	X			X	X	
Brazil						X			X
Bulgaria		X		X		X			X
Cameroon	X	X				X	X	X	X
Canada-Atlantic	X	X				X	X		
Chile			X	X			X		
China, P.R.	X	X	X	X	X	X	X	X	X
Denmark	X		X	X			X	X	
France	X	X	X	X		X	X		X
Germany			X			X	X		X
Hong Kong, SAR	X		X				X		
Ireland	X		X				X	X	
Israel									
Italy	X	X	X	X		X	X	X	X
Japan	X		X	X			X	X	X
Korea, South	X	X	X	X		X	X	X	X
Kuwait	X	X				X	X		X

…/…

TABLE 22.2 *(Suite)*

Country	Fish culture	Fish wild stocks	Mollusc culture	Molluscs wild stocks	Crustacean culture	Natural ecosystems	Public safety	Quality control of products	Recreational/ Tourist aspects
Malaysia			X		X	X	X		X
Netherlands			X	X	X	X	X		X
New Zealand	X	X	X	X			X	X	X
Norway	X	X	X	X			X	X	
Philippines–Bataan		X		X			X	X	
Portugal	X	X	X	X	X	X	X	X	X
Qatar						X	X		
Romania		X		X					X
Seychelles		X					X		
Slovenia							X		
Spain						X	X		X
Spain-Monteverdi	X		X	X		X	X	X	X
Sweden–Baltic							X		
Thailand	X		X	X			X		X
Tunisia			X	X			X		
Turkey			X	X			X	X	
UK–England & Wales			X	X			X		

…/…

TABLE 22.2 *(Suite)*

Country	Fish culture	Fish wild stocks	Mollusc culture	Molluscs wild stocks	Crustacean culture	Natural ecosystems	Public safety	Quality control of products	Recreational/ Tourist aspects
Uruguay		X	X	X		X	X	X	X
USA-Maine			X	X			X	X	X
USA-Delaware		X				X	X		X
USA-New Jersey						X	X		X
USA-New York State	X	X	X			X	X		X
USA-New York-Suffolk		X		X		X			X
USA-North Carolina						X	X		X
USA-Texas	X	X				X	X		X
USA-Washington							X		
Venezuela		X	X	X	X	X	X	X	X
Vietnam	X		X	X	X		X	X	

It is important that all routines for sampling, sample analysis, data analysis and storage be clearly defined and that the staff responsible for each step in the process have access to detailed descriptions of the relevant methodologies. It must be clear which institution/person is responsible for compilation/synthesis of the monitoring results and how the results of the synthesis are to be presented to the users of the programme. Pre-printed forms should be available to be filled in with the monitoring data as well as additional information on the sampling, such as location/position,

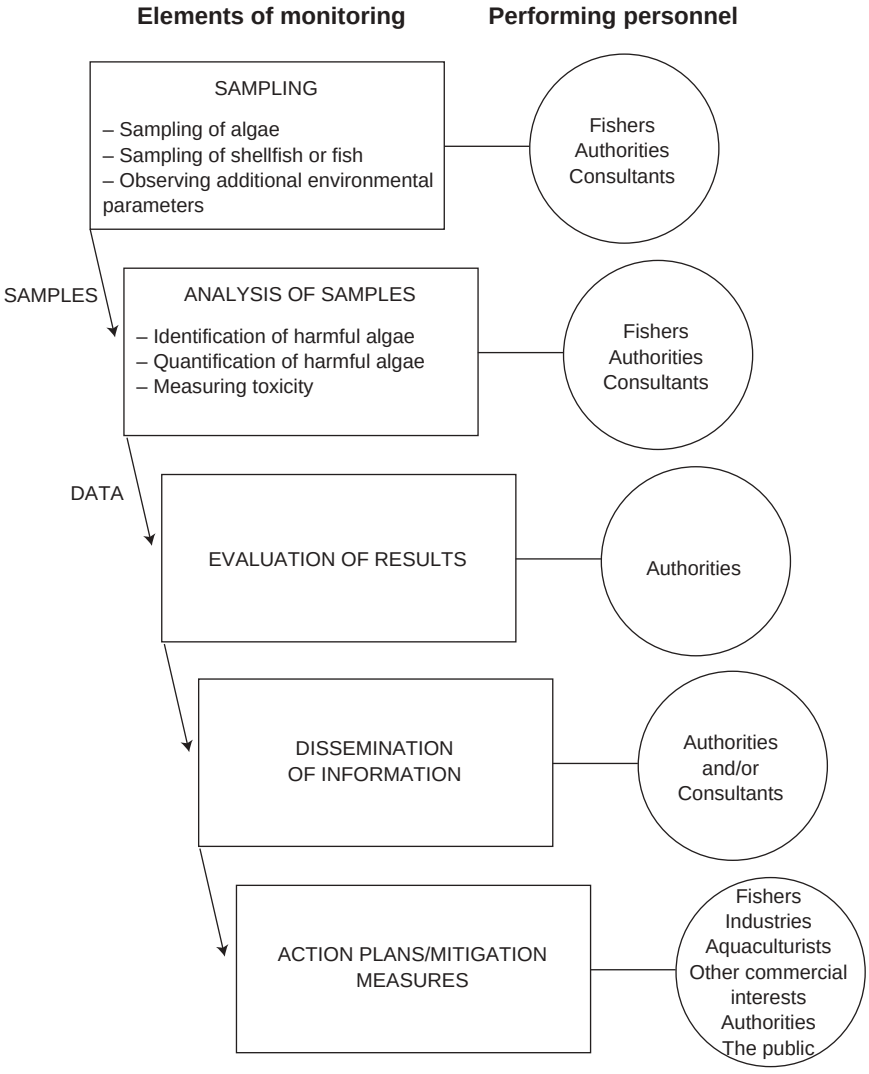

Figure 22.1
Theoretical design elements of a HAB monitoring programme indicating how information should be passed from one element to the next, and who is carrying out each process.

station name/number, and identification code for the staff responsible for the sampling. 'Raw' data from the monitoring programme should be kept in files, paper or electronic form for later reference and investigation. It is recommended that the data be stored in a computer database to facilitate data handling, quality assurance and

TABLE 22.3 An overview of the linkage between the resource to be protected from HAB effects, the potential HAB impact on the relevant resources and the basic parameters to be monitored in order to manage those resources

'Resource' to protect	HAB impact on resource	Monitoring parameters
A. Direct impact on humans		
Shellfish for human consumption	Toxicity due to accumulation of algal toxins in shellfish flesh	Algal toxins in shellfish flesh and occurrence of harmful algae
Fish for human consumption	Toxicity due to accumulation of algal toxins in fish meat	Algal toxins in fish meat and occurrence of harmful algae
Drinking water	Toxicity due to occurrence of algal toxins in surface water and desalination plants	Algal toxins in drinking water and occurrence of harmful algae in the source water
Recreational use of coastal waters/lakes/rivers	Intoxication due to exposure to algal toxins in water/aerosols/air Loss of aesthetic value due to discoloration of water Odour and smell due to degradation of algae	Harmful algae, algal toxins
B. Economic impact		
Wild fish stocks	Fish kills, stress	Harmful algae, fish behaviour, occurrence of dead fish
Aquaculture	Fish kills, stress, inhibit growth	Harmful algae, fish behaviour, occurrence of dead fish
C. Environmental impact		
Biota/ecosystems	Change of biological community structure due to kill off and/or inhibition of growth as a result of intoxication and/or oxygen deficiency	Harmful algae, species-specific behaviour, die-offs, lowered recruitment, etc.
D. Conservation impact		
Endangered/protected species	Kills/stress/inhibition of growth and reproduction	Harmful algae, behaviour of species of interest, die offs, inhibition of reproduction

analysis. Before data or results are released to the monitoring or management system, they should be properly checked by at least one person who did not perform the analysis. Once the results of the different analyses are available, it is important that well-defined routes for communication of the results be available in an official manual which defines:

- types of samples to be collected and sampling methodology;
- types of analyses to be performed;
- institution/individual responsible for collecting the samples;
- institution/individual responsible for analyses of the samples;
- how the data are archived and analyzed;
- quality assurance procedure;
- documentation (forms to be used);
- data analysis;
- data archiving;
- data presentation and evaluation.

An example of a well-designed HAB monitoring manual is the New Zealand National Marine Biotoxin Management Plan (Marine Biotoxin Management Board, 1996). The manual is set up so that pages are easily removed and replaced with updated versions. Material in the manual includes details of the administration of the national plan, methodological details, harvesting closure and re-opening procedures, methods for investigating toxic shellfish poisoning cases, product control, as well as a range of appendices with definitions, forms and other details. The New Zealand Monitoring Programme is described in detail as a specific case study in Chapter 23.

It is often the case that the personal experience of individuals who have been directly involved in the analysis of HAB data over many years allows those individuals to make forecasts or predictions of HAB incidents or transport. These individuals are extremely valuable to monitoring programmes, and every effort should be made to keep them involved in programme activities. Though non-quantitative, this type of empirical analysis is often quite accurate for predictive purposes.

Complementary marine environmental data are equally important in the analysis of already acquired HAB data as they are a basis for an early warning. Provided that the environmental data are available close to real-time, they may allow or support adjustments in HAB monitoring mode and associated action plans (for details see Anderson *et al*., 2001). Monitoring marine environmental conditions in relation to HABs can be carried out at different levels of temporal and geographical, as well as vertical and horizontal, resolution depending upon which kind of harmful algal bloom is to be monitored (see Chapter 21). Furthermore, depending upon the goal of the monitoring, it can include a range of biological, chemical and physical parameters (Table 22.4). The list is long and is meant only as a general guideline. Specific programmes may find that only a subset of these parameters is relevant to their goal.

22.2.2 Operational HAB monitoring – action plans and mitigation measures

HAB monitoring programmes must be dynamic, that is, they must be able to operate in different modes as a response/adaptation to changes in present HAB situations. Such responses could be increasing the frequency of sampling and/or the geographical coverage of the monitoring. Furthermore, the monitoring of toxicity in shellfish and fish can be intensified in case a HAB is observed.

TABLE 22.4 Examples of potential environmental parameters to be included in HAB monitoring programmes

Physical	Chemical	Biological
Temperature (vertical profile or remote sensing data)	Salinity (vertical profile)	Phytoplankton
Current speed and direction (vertical profile)	Oxygen content (vertical profile)	– Harmful species
Wind speed and direction	Chlorophyll (vertical profile or remote sensing data)	– All species
Light attenuation/turbidity	Nutrients:	Meso-zooplankton
	– Nitrogen	Proto-zooplankton
	– Phosphorous	Pelagic bacteria
	– Silicate	Fish
		Benthos
		Birds

The following monitoring modes are suggested:

- Normal/routine mode (to operate in non-HAB situations);
- Watch mode (to operate when a HAB is observed but in rather low concentrations or when low levels of algal toxins are detected in shellfish and/or fish);
- Alert mode (to operate when a HAB is observed in critical concentrations, when algal toxins are observed in concentrations close to or exceeding regulatory limits or where HAB effects on fish are observed).

The change of a HAB monitoring programme from one mode to another must be triggered by specific observations or combinations of observations already identified as triggering factors or triggering scenarios (Figure 22.2., Table 22.5).

Trigger 1:

- Changes in water temperature, hydrography, chlorophyll etc. (e.g. registered using remote sensing data as part of an early warning system);

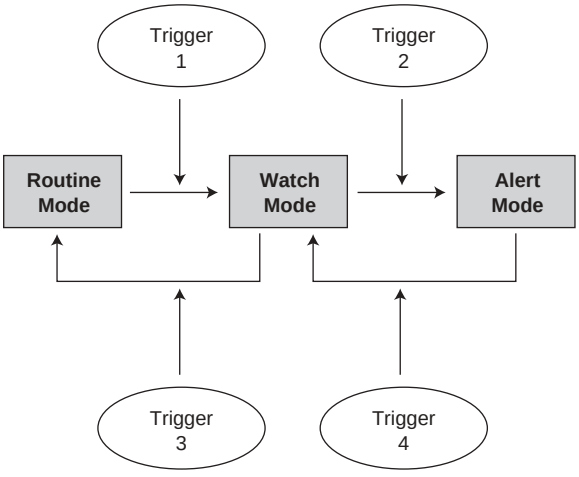

Figure 22.2
Generic action plan with 3 different monitoring modes including examples of triggering factors/scenarios responsible for change of monitoring modes.

- Algal toxins present but below a given regulatory limit;
- Harmful algal species present e.g. above a concentration considered to be of concern.

Trigger 2:

- Algal toxins above a given regulatory limit;
- Fish behaviour indicates intoxication.

Trigger 3:

- Harmful algae not present or the concentration is below a level considered to be of concern;
- Algal toxins not present.

Trigger 4:

- Algal toxins present but below a given regulatory limit;
- Fish behaviour normal.

TABLE 22.5 An overview of relevant monitoring HAB modes to protect various resources and the associated triggering observations for change in mode

Resource to protect	Triggering observations	Monitoring modes
A. Direct impact on humans		
Shellfish for human consumption	Concentrations of potentially toxic algal species or toxicity trigger changes in monitoring mode and management practice. The concentrations that trigger changes in monitoring mode are species-specific. *Example*: concentrations of *Alexandrium tamarense* exceeding 500 cells l^{-1} or *Karenia brevis* exceeding 5000 cells l^{-1} might result in closure of shellfish harvest.	Watch mode
	Concentration levels of toxins in shellfish/fish meat trigger monitoring mode and management practice. *Example*: 80 µg PSP toxin/100 g shellfish meat should result in closing of harvest, as the shellfish are not considered safe for human consumption.	Alert mode
Fish for human consumption	CFP toxins present critical concentrations in fish meat. Fish are not considered appropriate for human consumption. (At present no official guidelines exist for CFP or other algal toxins in fish meat.)	Alert mode
Drinking water	Freshwater with concentrations of microcystin exceeding 1 µg l^{-1} is not considered appropriate for human consumption (WHO, 1990). (At present no guidelines exist for freshwater produced at desalination plants or for the intake water to the plants.)	Alert mode

…/…

TABLE 22.5 *(Suite)*

Resource to protect	Triggering observations	Monitoring modes
A. Direct impact on humans		
Recreational use of coastal waters/lakes/ rivers	Observation of algal blooms/scum is in general considered to impose an increased risk for recreational human users of coastal waters/lakes/rivers (WHO guideline, in prep.). In the case of blooms of *Karenia brevis* and *Pfiesteria* spp. the public is warned.	Watch mode – the public is warned not to swim etc.
B. Economic impact		
Wild fish stocks	Blooms of *Chattonella*, *Karenia mikimotoi*, *Karenia brevis*, *Chrysochromulina* spp., *Heterosigma akashiwo* and other icthyotoxic species.	Watch mode
Aquaculture	In the case of blooms of *Chattonella*, *Karenia mikimotoi*, *Karenia brevis*, *Heterosigma akashiwo*, *Cocchlodinium polykrikoides*, *Chrysochromulina* spp. *Chaetoceros* spp. etc. Monitoring/ management practice can be changed in different ways depending on the blooming species and the observed bloom concentrations (see Chapter 24)	Watch mode Alert mode
C. Environmental impact		
Biota/ecosystems	In the case of blooms of *Chattonella*, *Karenia mikimotoi*, *Karenia brevis*, *Chrysochromulina* spp. monitoring of different ecosystem components might be initiated. This might also be the case if oxygen deficiency is observed as a result of non-toxic HABs, e.g. a *Ceratium* bloom	Watch mode
D. Conservation impact		
Endangered/protected species	Blooms of relevant harmful species. In the case of blooms of *Karenia brevis*, Florida manatees might be transferred to sheltered areas	Watch mode or alert mode

In the case of HAB monitoring of shellfish, the occurrence of toxic or potentially toxic species of algae can be used to trigger testing for algal toxins in the shellfish meat, which is the primary parameter used to manage the harvest of shellfish. Furthermore, the occurrence of potentially toxic species can result in increasing the frequency of sampling of algae and shellfish for analysis (Watch mode). If the concentrations of toxic or potentially toxic algae exceed specific concentration limits,

shellfish harvesting can be closed in the specific harvest areas (Alert mode). In these cases the monitoring of potentially toxic or toxic species is used as an early warning that algal toxins might soon accumulate in the shellfish in concentrations above the given regulatory limits. This kind of monitoring practice is the case e.g. in Denmark, Norway, New Zealand, Spain and Vietnam (Andersen, 1996; IOC, 2001). Detection of low concentrations of algal toxins (below the critical limit) in shellfish meat can also be used to trigger increased sampling (Watch mode). The observation of algal toxins above critical limits can be used to close shellfish harvest (Alert mode), as is the case in most countries with commercial shellfish harvest (Andersen, 1996; IOC, 2001).

In the case of HAB monitoring in relation to aquaculture of fish/crustaceans, the observation of HAB species in low concentrations might result in a suggestion to fish farm operators to stop feeding their fish (Watch mode), whereas high concentrations might result in the towing of net pens to safer areas (Alert mode). (See Table 22.5 for more examples of triggering observations and Chapters 24 and 25 for more details.)

Forecasts can be made which define risk-zones, such as areas with a high incidence of toxic outbreaks or, conversely, areas where harmful blooms are rare. Site selection of aquaculture facilities often requires careful analysis of long-term monitoring data to identify sites with a low risk of HAB events. Here again, the experience of long-time workers in the monitoring programme can provide valuable justification for avoiding or selecting a particular site.

Each monitoring mode should have an associated action plan. When a monitoring programme is shifting from Normal to Watch mode, only minor adjustments to the monitoring action plan may be required. On the other hand, if the HAB situation is critical, Alert mode monitoring must be initiated and pre-approved action plans (mitigation/control) initiated to protect the resources or minimize damage otherwise caused by the HAB. Action plans are likely to be resource-specific. Action plan elements for shellfish affected by HABs include:

- Increase of sampling frequency and geographical coverage
- Closure of shellfish harvest
- Withdrawal of shellfish from the market, depuration of shellfish before they are used for human consumption
- Communication programmes to inform the public that commercial shellfish might be toxic and that recreational collection of shellfish is prohibited/not advised.

Such action plans are initiated based upon the observation of algal toxins above the given regulatory limits in most countries e.g. in Galicia, Spain, Figure 22.3. The Spanish action plan involves multiple monitoring modes (Plan A–D) which are implemented with the observation of one or several triggering factors such as the detection of toxic species in low concentrations, calm weather conditions etc. In some countries, e.g. New Zealand, Norway and Denmark, closure of shellfish harvest and/or advice to the public not to collect shellfish can also be initiated due to algal concentrations above limits.

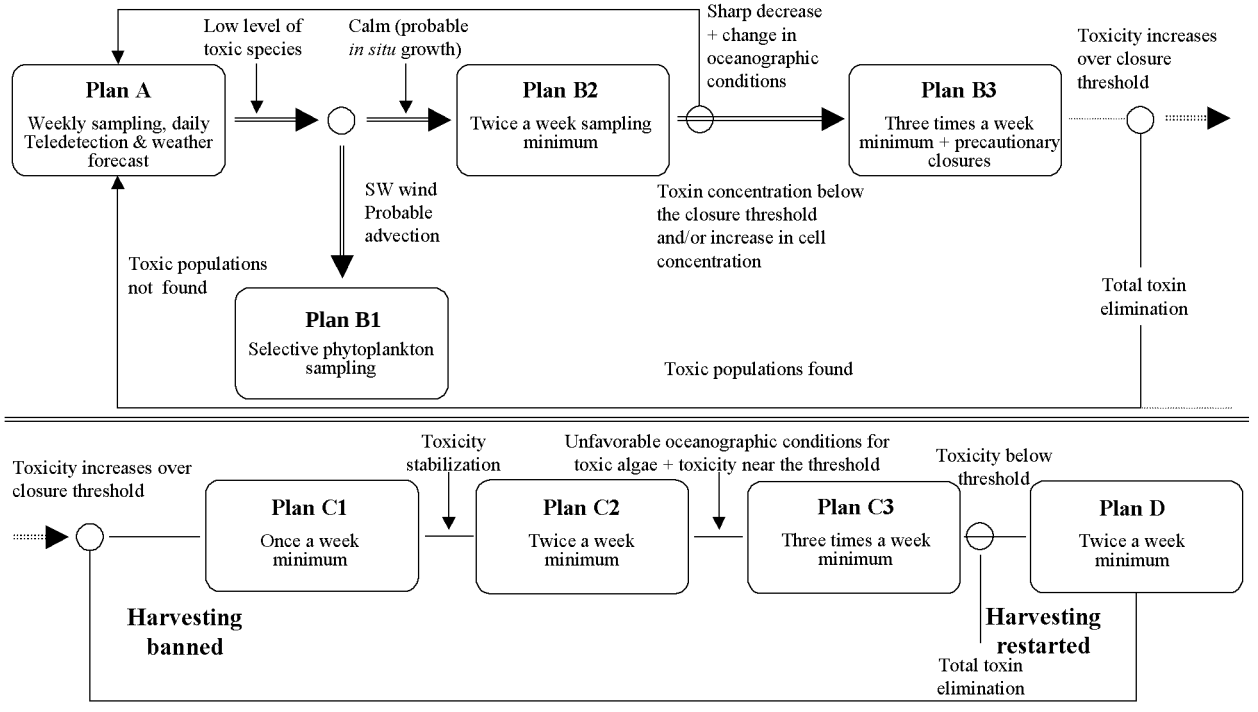

Figure 22.3
Action plan for the biotoxin monitoring programme in Galacia, NW Spain. The different monitoring modes are denoted as Plan A, B, B1 etc. Triggering factors are shown as thin arrows pointing to the lines linking one plan to the next. (Note: the figure starts left at the upper panel and continues at the left of the lower panel, from Mariño *et al*., 1998.

Action plans for aquaculture operations include a range of mitigation measures such as:

- Stoping feeding the fish
- Preparing for and/or conducting pre-emptive harvest
- Moving the culture pens into waters with less risk of HAB
- Applying perimeter skirts to culture pens
- Aeration
- Potentially suppressing the HAB by adding materials to disperse or suppress the algal bloom (e.g., clays, or other, see Chapter 25).

Action plans for recreational use of coastal waters include measures such as:

- Advising the public to stay out of the water
- Advising the public to shower properly following swimming during certain types of HABs
- Advising the public to avoid inhaling sea spray.

Action plans to rescue/save endangered/protected species from HABs may include measures such as:

- Ill or affected animals can be transferred to protected areas during HABs
- Bloom mitigation measures (e.g., clay dispersal) if the situation is severe and the resource to be protected is sufficiently important.

Action plans for protection of the general environment from the effects of HABs usually do not include short-term measures, but may include actions such as minimizing the run-off of nutrient-rich water from land (eutrophication) which, in the long term, may reduce the risk of certain types of HABs.

22.2.3 Distribution of information to users

After appropriate quality assurance, data and results can be distributed to the users of the monitoring programme by telephone, telephone answering machine, fax, e-mail and the Internet. The use of the Internet to distribute HAB monitoring data is common in many countries/regions, e.g. in Denmark, Germany, Norway, Hong Kong SAR, Ireland, Italy, Sweden, and USA. Most of these web pages contain general information on HABs as well as updated information on the current algal situations. Some pages also include recommendations to recreational shellfishermen and information on the present situation of the commercial shellfish harvest. Geographic information system (GIS) tools can be very useful to present and evaluate HAB information. Additionally it is highly recommended that status reports be made available on local/global networks or as easily accessible reports on the Internet.

Public information is a natural part of a HAB action plan. Information material should be available and communication strategies involving different media (TV, radio, newspapers, Internet, callers, posters, booklets etc.) should be prepared.

22.2.4 Organization and structure of HAB monitoring programmes

The structure of a HAB monitoring programme can become rather complicated depending upon the number of institutions involved in the individual analysis procedures at each level in the network. Nevertheless, the structure of HAB monitoring programmes must be kept as simple as possible to facilitate a rapid and uncomplicated flow of information between the individuals and institutions involved. Responsibilities for specific elements of the programme must be clear to all individuals involved.

The operational structure should be well documented in the form of a report distributed to all users, containing information on which institutions are involved (addresses, phone and fax numbers, e-mail addresses etc.), the responsible persons in the different institutions (addresses, phone and fax numbers, e-mail addresses etc.) and a clear description of which tasks each institution/person is responsible for. This report should be periodically reviewed and updated.

In most cases (85%) of the existing HAB monitoring systems, the institution/organization that initiates a HAB monitoring programme is also responsible for carrying out the monitoring operations (Andersen, 1996). In some cases the management structure as well as the conduct of the HAB monitoring programmes is advised by or has to refer to a task force if the programme is to be changed. This is the case in New Zealand, where the Marine Biotoxin Management Board must confirm and approve any changes in the HAB monitoring system, whereas in Denmark a HAB task force under the Veterinary Service discusses relevant issues and suggests changes in the monitoring and management procedures which subsequently must be approved by the Veterinary Service. If an unusual HAB occurs in Danish waters, the Danish HAB task force will be summoned to discuss the current situation and give advice to the Veterinary Service on what to do. This kind of administrative unit for technical advice is also currently part of the Japanese, Hong Kong, and Philippine HAB management system. In Japan a 'Consultative Committee' provides the technical advice, whereas it is called 'National Red Tide Committee' in the Philippines (Andersen, 1996; Anderson, *et al*. 2001).

Most HAB monitoring programmes rely on different official authorities and/or universities for sampling of algae and shellfish for toxicity analysis (Andersen 1996, IOC 2001). Private consultancy companies can also be involved in HAB monitoring programmes. For example, in the state of California, sampling of algae and shellfish is carried out at fixed stations at weekly intervals by local companies, organizations etc. on a voluntary basic under the guidance of the State of California Department of Health Services, which is also responsible for analysing the samples. In Denmark the fishermen carry out the sampling of algae and mussels, and private consultancy companies analyse those samples. The consultancy companies report to the Ministry of Food, which is responsible for the management decisions (Andersen 1996).

As the organization of a HAB monitoring and management programme depends heavily on the country-specific structures it has to interact with and depend on, it is difficult to provide a generic description here. Figure 22.4 illustrates a hypothetical master plan for a HAB monitoring and management programme.

The complex monitoring/management structure for a multi-resources management system (Figure 22.4) is designed to co-ordinate HAB monitoring in relation to a whole range of resources including fish (wild, aquaculture and imported), shellfish (wild, aquaculture and imported), recreational/tourist aspects, environmental impact and conservation impact. The generic master plan relies on co-operation and co-ordination of the monitoring and management effort carried out by a range of government agencies. Furthermore it assumes that observational information can be obtained from fish farmers and other participants as well as from neighbouring countries or regions. An Operational Technical Team (OTT) composed of technical representatives of the involved agencies collects all monitoring information. The OTT is responsible for initiating action plans and changing of monitoring modes. A HAB Task Force (composed of higher-level officials in the relevant agencies) must agree with any changes in the monitoring programme. The

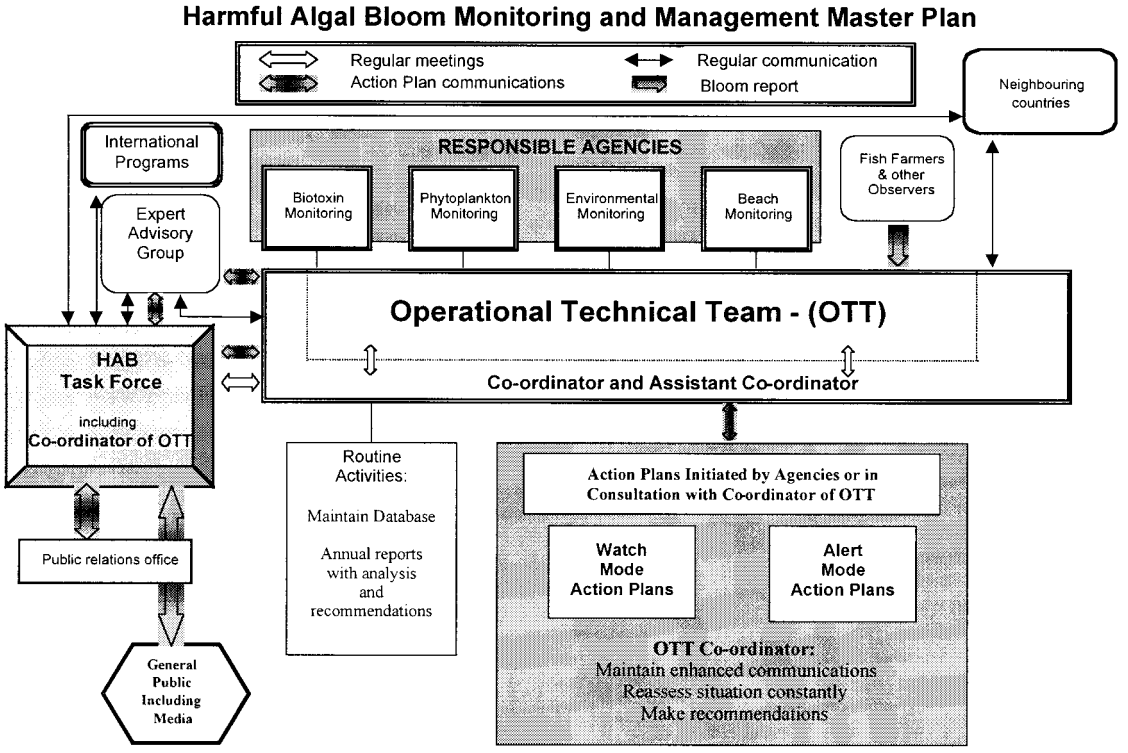

Figure 22.4
One hypothetical structure for a multi-resource HAB monitoring programme.

HAB Task Force can consult the Expert Advisory Group for scientific/technical advice. All public information is to be communicated by a selected member of the HAB Task Force in cooperation with the relevant public relations office to minimize confusion during a HAB. If more than one member or agency communicates to the public, confusion can often occur, so it is best to assign this role to a single, well-informed individual.

The following examples are offered to demonstrate the management structure used in Denmark and Norway. See Chapter 23 for a detailed case study from New Zealand.

Example 1. *Simple monitoring/management structure of one single resource*. The Danish HAB monitoring programme for Danish mussel fisheries is constructed to manage HABs in relation to a single resource – wild stocks of mussels (Figure 22.5). The official regulations concerning sampling and quantification of toxic phytoplankton and algae toxins in relation to the Danish mussel fisheries are given in European Union order no. 202 'Laying down the health conditions for fishery, handling, production and placing on the market of bivalve molluscs, 15 April 1993' and the guidelines no. 7000 of 27 April 1993 and no. 13220 of 28 February 1995. The Danish legislation implements the EEC Council Directive of 15 July 1991 'Laying down the health conditions for the production and the placing on the market

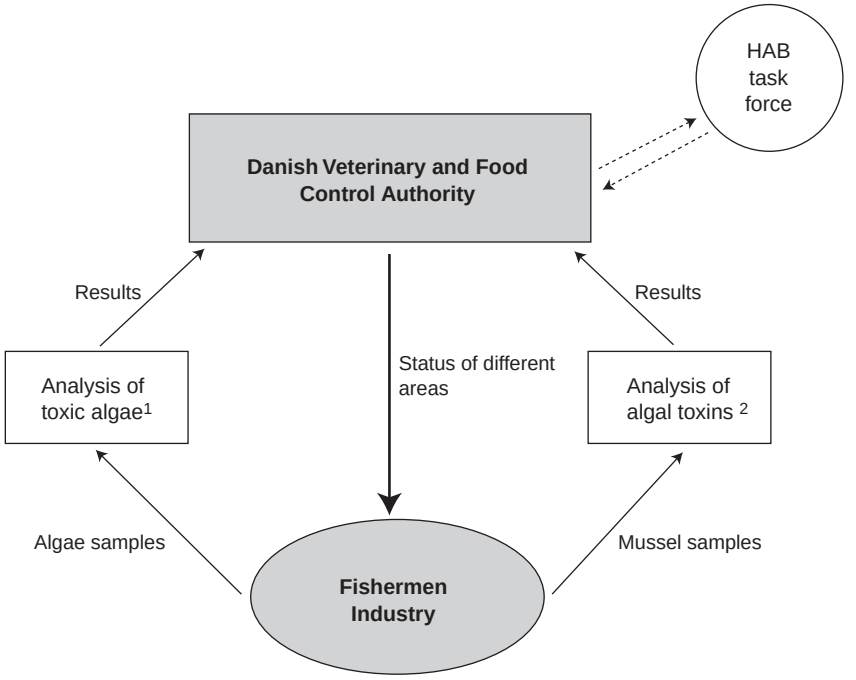

Figure 22.5
The structure of a simple single-resource HAB monitoring programme — The Danish HAB monitoring programme in relation to the Danish mussel fisheries.

of live bivalve molluscs', 91/492/EEC, with later amendments. The monitoring programme works as follows. Samples of algae and mussels are collected by the fishermen and are sent to private consultancy laboratories. The results of the analysis are forwarded directly by the laboratories to the Danish Veterinary and Food Control Authority, which inform the fishermen and industries on the status of each fishing area. At least once a year the Danish HAB task force meets to review the on-going monitoring and management procedures and to exchange new findings nationally and internationally. The Danish Veterinary and Food Control Authority implements the recommendations of the HAB task force. The monitoring programme is not coordinated with, nor does it rely on information from other Danish monitoring programmes, but data are exchanged between the local environmental authorities and the HAB monitoring programme.

Example 2. *A multi-resource monitoring/management structure*. The Norwegian HAB monitoring programme is designed to protect the significant marine aquaculture enterprises, shellfish cultures as well as recreational collection of shellfish. The monitoring programme covers most of the coastal waters and fjords of Norway. Information on the occurrence of HABs is gathered from different sources such as a "fish farm observation network" covering 40 sites along the Norwegian coast, including fish farmers, aquaculture research stations, lighthouses and mussel farmers. A total of 80 stations are currently sampled in Norwegian coastal waters, covering the Norwegian coastline from the Swedish border in the South to the Russian border in the North. Samples for monitoring of harmful algae are collected weekly by a number of fish farmers, mussel farmers and the Norwegian Food Control Authority as well as research institutions. If algal concentrations are high, based upon Secchi disk transparency measurements and watercolour, fish farmers will collect additional samples. Guidelines for sampling as well as Secchi depth measurement are provided to the fish farmers. Furthermore, the Norwegian Food Control Authority, as well as offshore moorings known as SEAWATCH-buoys, collects oceanographic data.

All data are collected and compiled by a consultancy company, which is responsible for advising fish farmers if an HAB should occur. The shellfish data are evaluated by, and are available from, the Norwegian Food Control Authority. Furthermore the complete data set is used to evaluate the HAB situation in Norwegian coastal waters on a weekly basis and the result of the evaluation is published on the internet as 'Algeinfo' by the Institute of Marine Research in co-operation with the consultant, the Ministry of Fisheries, and the Norwegian Institute for Water Research (Figure 22.6).

In the Norwegian programme, it is considered very important that the fish farmers themselves take immediate action if a fish-killing bloom is reported in an area, or if they observe the fish beginning to behave abnormally. The action that must be taken to reduce losses involves different mitigation measures, which are carefully planned and tested in advance.

In the case of shellfish, the Norwegian Food Control Authority regulates the harvests from specific aquaculture sites and the public is informed on the HAB situation and the risk of collecting toxic shellfish via the 'Algeinfo' web page as well as through a web page run by the Norwegian Food Control Authority.

The Norwegian monitoring programme is periodically revised based upon experience in Norway and internationally. Over the years, revisions have involved moving sampling stations and/or increasing the number of stations to improve the

coverage of the monitoring, as well as updating and adding new names to the list of HAB species in Norwegian waters.

22.3 Improving HAB Monitoring and Management

To improve existing or future HAB monitoring and management and the related action plans, systematic collection of qualitative and quantitative data on the occurrence of HAB species, the algal toxins and effects of HABs, as well as epidemiological studies are needed. Based on such data sets the monitoring effort can be optimized with respect to its ability to actually forecast/detect developing HAB events, the effectiveness of management and mitigation during HAB events, and cost efficiency. Furthermore, improved data acquisition and analysis are pre-requisites for future improvements of HAB forecasts and control. This might include using satellite remote sensing data, in-situ buoy measurements in combination with physical and biological modelling, as well as improved analysis and evaluation of the data available to identify potential bloom scenarios and bloom triggers. This often goes beyond the resources and mandate of national monitoring programmes. This is why basic research is often necessary to support and augment monitoring programmes.

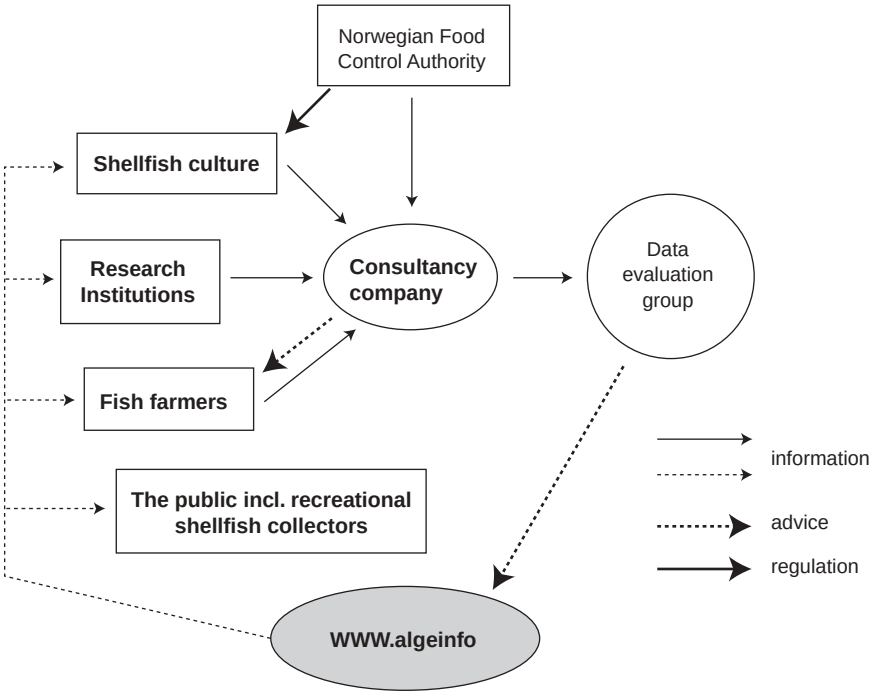

Figure 22.6
The structure of the Norwegian multi resource HAB monitoring programme covering marine fish farms and shellfish cultures as well as recreational collection of shellfish.

At the international level, research programmes such as GEOHAB have established the goal to improve our ability to predict and thereby improve HAB management and mitigation GEOHAB, 2001).

Other aspects, which require regional and international monitoring cooperation, are the potential spreading of HAB species over long distances via ballast water of ships, with live seafood, etc., and the cross territorial effects of cultural eutrophication, which can be of importance especially for the high biomass HAB species. Coordination within countries/regions as well as coordination/sharing of information between countries/regions is also important because HABs are phenomena that

Figure 22.7
Map from the Norwegian Internet Homepage 'Algeinfo' showing the sampling locations and a legend box explaining the different symbols used on the map. Note that the map is from a winter situation (February 2002) where no HABs occurred.

cross territorial boundaries. Local management plans may become even more efficient and able to provide an early warning if exchange of information and data are facilitated across national or regional borders.

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23

Role of phytoplankton monitoring in marine biotoxin programmes

K. Todd

23.1 Introduction

Many countries monitor for biotoxins in shellfish in order to meet both domestic food-safety requirements and export standards for shellfish products (Chapter 24). To be successful, monitoring programmes are required to provide an early warning of the potential for biotoxin contamination in shellfish and to limit the harvesting and unnecessary disposal of shellfish due to toxin levels that exceed food-safety regulations. Programmes therefore need to assess the causative phytoplankton organisms in the water column, as well as toxins in the shellfish flesh.

Such programmes require good oceanographic knowledge of the area to be monitored, trained sampling officers, skilled phytoplankton taxonomists and experienced chemical analysts. A good communication network is essential for the rapid dissemination of results and sharing of information and knowledge among all parties involved in biotoxin management and research.

23.2 The New Zealand experience

Harmful algae are monitored as part of marine biotoxin management in New Zealand for two main reasons: (a) quality assurance of shellfish products being exported to trading partners; and (b) protection of public health from marine biotoxins from both commercially harvested and recreationally gathered shellfish.

New Zealand had its first experience of marine biotoxins in the summer of 1992–1993, with the closure of the shellfish industry for several months following a neurotoxic shellfish poisoning (NSP) outbreak (Trusewich *et al.*, 1996). Prior to this event, the experience of the effects of harmful algae had been limited to problems caused by ichthyotoxic raphidophyte and dinoflagellate species to sea-farmed finfish. Since 1993, New Zealand has established, and continues to refine, marine biotoxin management strategies to incorporate new technologies and knowledge.

23.2.1 New Zealand Marine Biotoxin Monitoring Programme

New Zealand is one of the few countries in the world where most of the toxin groups (PSP, DSP, ASP and NSP – see Table 23.1) are found; therefore the programme is set up to monitor for all potentially toxic phytoplankton species and all the toxin groups. Approximately 70 sites from around the New Zealand coastline have sea-

water samples collected from them weekly for phytoplankton analysis. The sites have been selected in order to give the best possible correlation between phytoplankton and toxins. Site selection is based on historic phytoplankton and toxin data, oceanographic knowledge and general suitability (e.g. accessibility, safety, shellfish resources, etc.). For example, exposed surf beaches are not routinely sampled due to their rough nature, which makes it difficult both to collect safely a sample and to collect one free from debris that makes microscopic analysis difficult.

Each week, trained health or industry representatives collect seawater samples using either Van Dorn or hose samplers. Van Dorn samplers are used to collect samples at discrete depths (usually at 3 m intervals) through the water column. The hose sampler is used to collect a sample through the water column, usually to a depth of 15 m. The samples are generally collected from a boat, with each sample being poured into a bucket rinsed with seawater and two 100 ml subsamples taken from the bucket. One of these subsamples is immediately preserved with

TABLE 23.1 Phytoplankton levels used in New Zealand to trigger further sampling/testing of shellfish for marine biotoxins (values in cells per litre). These are applicable to both commercial and recreational harvesting and relate to composite samples

Phytoplankton species	Toxin group	Level in sample to trigger flesh sampling	Industry 'voluntary' closure pending flesh-testing results	Issue public health warning
Alexandrium catenella	PSP	100	500	5,000
A. minutum	PSP	100	500	5,000
A. ostenfeldii	PSP	100	500	5,000
A. tamarense	PSP	100	500	5,000
Gymnodinium catenatum	PSP	100		
Pseudo-nitzschia spp. (>50% total phytoplankton)	ASP	50,000	200,000	na
Pseudo-nitzschia spp. (<50% total phytoplankton)	ASP	100,000	500,000	na
Gymnodinium cf. breve	NSP	1,000	5,000	5,000
Dinophysis acuta	DSP	500	1,000	na
D. acuminata	DSP	1,000	2,000	na
Prorocentrum lima	DSP	500	1,000	na

PSP: paralytic shellfish poisoning toxins; ASP: amnesic shellfish poisoning toxins; NSP: neurotoxic shellfish poisoning toxins; DSP: diarrhetic shellfish poisoning toxins.
Source: National Marine Biotoxin Management Plan, New Zealand (modified).

four drops of Lugol's iodine while the other remains unpreserved. The samples are kept dark and cool (but not refrigerated) and are freighted overnight to the laboratory. For each site, a form detailing time of sampling and general environmental conditions is completed and dispatched with the samples. The discrete depth samples are subsampled (10 ml) at the laboratory and pooled to form an integrated sample. This integrated sample and the hose samples are pipetted (10 ml) into Utermöhl sedimentation chambers and are left to settle for 4 hours prior to analysis using inverted microscopes. The potentially toxic microalgae are identified and enumerated, and the results recorded and reported to the health and/or industry representatives responsible for the management of that area within 24 hours of receipt of sample.

In tandem with phytoplankton sampling, shellfish samples are collected and sent to the laboratory, where they are analysed for toxins. Depending on the management plan, an area may have samples analysed weekly for all toxins; other areas may have samples analysed for certain toxins at different intervals, such as fortnightly or monthly; and yet other areas may have shellfish analysed for all toxins once a month, which acts as an 'audit' of the phytoplankton monitoring. If designated species of phytoplankton are present in numbers above acceptable limits, then a shellfish sample will also be collected and tested for the appropriate toxin. If the shellfish sample contains toxin above the regulatory limit, the area is closed for harvesting and the general public is warned not to gather shellfish from that area. While a closure is in place, phytoplankton and shellfish sampling and testing continue on a weekly (or more frequent) basis. When toxin levels decrease below the regulatory limits, the area can be reopened once the reopening criteria have been met (see Fig. 23.1).

Programmes are in place for the management of marine biotoxins in both commercial and non-commercial (recreational) areas. They are funded separately by the commercial shellfish aquaculture industry (for both commercially cultured and wild stocks); and by the Ministry of Health (for non-commercial areas). Both programmes work on the principle of phytoplankton monitoring being the first tier in a two-tier system, and providing early warning of potential contamination from marine biotoxins. Each commercial area has a health representative who is involved in the administration and management of the area, and who is responsible for opening and closing the area as required.

23.2.1.1 *Use of trigger levels for phytoplankton*

In order for phytoplankton monitoring to work as an early warning system, trigger levels or action levels need to be in place that ensure further testing of shellfish is undertaken when these levels are met or exceeded. Levels should be set conservatively for known toxin-producing species, hence it is important to know which species are present in the area and what toxins are produced by these species. The levels used in New Zealand trigger the following actions: (a) collect shellfish samples for toxin testing; (b) instigate a 'voluntary' closure by industry, pending flesh-testing results (i.e. stop industry harvesting until flesh results are available; and (c) issue public health warnings (see Fig. 23.1). These trigger levels are based on local and international experience. They can be altered as more research is undertaken and knowledge of both phytoplankton and toxins increases. Andersen (1996) summarizes cell concentrations used internationally to trigger further monitoring or harvesting restrictions.

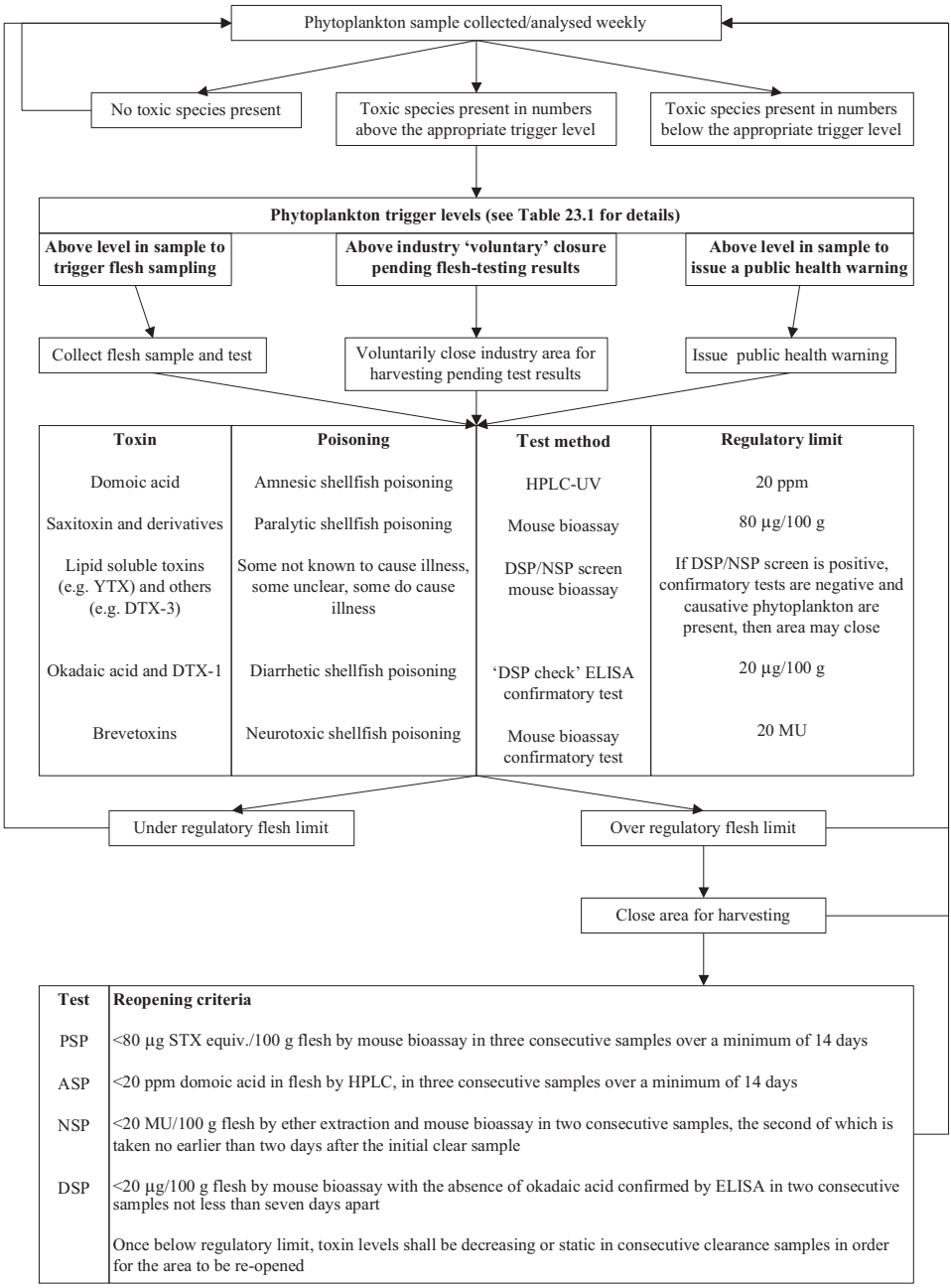

Figure 23.1
New Zealand Marine Biotoxin Monitoring Programme.
Source: National Marine Biotoxin Management Plan, New Zealand (modified).

For the PSP-producing *Alexandrium* species such as *A. fundyense*, *A. minutum*, *A. ostenfeldii*, and *A. tamarense*, limits range from detection to 10^3 cells per litre; for *A. catenella*, the limit is as high as 10^4 cells per litre in some countries; and for *Gymnodinium catenatum*, the limits range from presence to 2×10^3 cells per litre. In New Zealand, all PSP-producing species have limits set at a presence in samples (100 cells per litre).

For the DSP-producing *Dinophysis* species the majority of cell concentration limits range from 100 cells per litre to 10^3 cells per litre, with the exception of *D. sacculus* and *D. acuminata* in the Valencia region in Spain where they may reach 10^7 cells per litre before action is taken. In New Zealand the two main *Dinophysis* species are *D. acuta* and *D. acuminata*, which have levels set at 500 cells per litre and 1000 cells per litre, respectively before action is taken. For the other DSP-producing species, *Prorocentrum lima*, the limit ranges from detection to 500 cells per litre; in New Zealand 500 cells per litre is used.

For the NSP-producing *Karenia* (*Gymnodinium*) *brevis*, New Zealand has adopted the 5000 cells per litre limit used in Florida for instigating a closure, but has also implemented a 1000 cells per litre limit for shellfish testing.

For the ASP-producing *Pseudo-nitzschia* species, New Zealand has adopted limits based on the percentage of cells present in samples and does not distinguish between groups (e.g. *Pseudo-nitzscha seriata* group, *P. delicatissima* group). Limits used around the world range from 10^3 cells per litre to 2×10^5. In New Zealand, when *Pseudo-nitzschia* species comprise less than 50% of the total cells, the limit is 10^4 cells per litre; and when greater than 50% of the total cells, the limit is 5×10^3 cells per litre.

These regional differences in limits demonstrate the necessity of having toxicity information available. The establishment of a culture collection of phytoplankton is an invaluable tool for this purpose. Whenever there is a bloom of a toxic or potentially toxic species, or a previously unrecorded species is found, culturing of isolates is advisable. This provides an invaluable source of material for further research work, including research into the production of toxins. For example, *Protoceratium reticulatum* was found to be the producer of yessotoxin in New Zealand following culturing and testing of this species during and following a bloom (Mackenzie *et al*., 1997).

A culture collection can also be used as a source of toxin standards. Internationally there is a shortage of some standards, which has a flow-on effect on the ability to test for these toxins in shellfish or to do further research into toxicology. Not all species are easy to culture, so for the production of some standards it is practicable to collect material in bulk during a bloom event. One of the key factors in running a successful phytoplankton monitoring programme is having trained and skilled technicians analysing the samples. The culture collection is an invaluable tool for training analysts in the identification of phytoplankton and for comparing those species that are difficult to identify.

New Zealand's programme is constantly being improved with the introduction of new technology and knowledge. It is useful to have research organizations and commercial laboratories working closely together in order to ensure that this happens. By doing so, New Zealand has been able to introduce technology such as whole-cell genetic probes (Miller and Scholin, 1996; see also Chapter 5) into the monitoring programme to aid in the identification of *Pseudo-nitzschia* species, which allows better risk assessments to be given to shellfish harvesters. Ongoing

research involving culturing and testing of New Zealand isolates of *Pseudo-nitzschia* to establish toxicity supported this work (Rhodes *et al.*, 1998) and allowed the integration of gene probes into the New Zealand programme.

23.3 Phytoplankton monitoring and flesh monitoring

Phytoplankton and flesh monitoring both have advantages and disadvantages, and some circumstances may favour the use of either one or the other method, but preferably both. For example, the decision may be based on the sampling site selected, the availability of analytical services or the availability of funding.

23.3.1 Phytoplankton monitoring

23.3.1.1 *Advantages*

Phytoplankton analysis can be more cost-effective than shellfish testing. For example, in New Zealand, the cost of analysing a phytoplankton sample for all potential toxin producing species is approximately a quarter of that of a shellfish sample. Analysis is rapid, with results available within 24 hours of the sample being received at the laboratory, compared with two to three days for completion of some toxin tests. This allows harvesting decisions and area closures to be made conservatively and sooner, with an assessment of the risk of potential toxin contamination. Phytoplankton analysis is not restricted to those species which are known to produce toxins; it also warns of other potential problems such as aerosol effects of cells breaking up in surf or ichthyotoxic species likely to be of concern to finfish farmers.

Regular monitoring of phytoplankton gives early warning of the potential for toxins in shellfish and contaminated shellfish can then be left in the water to depurate naturally. Phytoplankton monitoring also gives a better overall understanding of the environment that shellfish live in, including which species are beneficial to their growth; it can also help to explain unusual toxin results, such as identifying previously unknown producers of toxin.

23.3.1.2 *Disadvantages*

Sampling for phytoplankton provides a sample taken at one point in time and space, therefore site selection is critical when establishing a monitoring programme. Knowledge of the currents and tides in an area is required, as is knowledge of the biotoxin history of the area. Phytoplankton-monitoring programmes require highly trained and skilled phytoplankton taxonomists who are able to identify and distinguish confidently between many species of phytoplankton and recognize those that are of concern.

23.3.2 Shellfish meat monitoring

23.3.2.1 *Advantages*

Sampling and testing of shellfish essentially provides a ‘cumulative’ sample of what has been happening in the water over time as the shellfish retain the toxin in their flesh. Testing the shellfish means that the product that is eventually consumed is tested. This increases consumer confidence that the product is safe. Flesh testing is the internationally accepted method for acceptance of shellfish to markets.

23.3.2.2 *Disadvantages*

Shellfish testing is more costly than phytoplankton analysis and needs specialist chemical analysts. Generally the analysis takes longer to complete than that of phytoplankton, which can delay management decisions on harvesting and closing of areas. Some toxin standards are difficult to obtain internationally, which affects quantification of toxins in shellfish. Once shellfish are contaminated with biotoxins and have been taken out of the water, the depuration of toxins is not viable and the contaminated product requires disposal.

23.3.3 A combination programme

By using both phytoplankton and flesh monitoring, it is possible to take full advantage of both methods. Because phytoplankton provides an early warning of potential biotoxin contamination, this can act as the first tier in a two-tier programme, with samples being analysed from the same sites at a regular frequency (at least weekly). These results will then be available to allow harvesting and closure decisions to be made within a short timeframe. The setting of levels to trigger further actions, such as testing flesh samples, helps in making these decisions. In conjunction with this, shellfish testing acts as the second tier, being performed both independently of the phytoplankton monitoring and as a result of the phytoplankton results. This ensures an early warning of potential contamination, the shellfish can be left to depurate naturally in the water when they become contaminated and the actual product that is consumed is tested in order to meet food-safety standards.

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24

Management of shellfish resources

M. L. Fernández, S. Shumway and J. Blanco

24.1 Introduction

Molluscan shellfish aquaculture has experienced a dramatic worldwide increase over the last decade. Although the species cultured, the farming and harvesting techniques, and the local consumption habits vary depending on the geographical area, coastal production areas are regularly affected by a range of toxin-producing phytoplankton that even in low concentrations can contaminate shellfish, causing human illnesses and severe economic losses to industry.

There have been many attempts to reduce the impact of phycotoxins on shellfish aquaculture. Knowledge of the rates of intoxification and detoxification of filter-feeding shellfish is important because selection of shellfish species that accumulate low levels of toxins and show rapid depuration could minimize the effects of toxic outbreaks. Once a shellfish product is contaminated, mitigation strategies are relatively limited; most countries have implemented monitoring programmes to protect public health and reduce economic impacts. Topics covered by these programmes depend on the country but may include monitoring of oceanographic variables (weather, currents, nutrients), toxic microalgal species and toxic content of seafood. Toxicity is determined by a range of testing procedures; the results are compared with regulatory limits to prevent unsafe products being placed on the market. Some of these programmes have received the status of official regulations laying down requirements for production areas and marketing, as well as methods and limits for use as sanitary control. When reviewing existing regulations, discrepancies in methods and limits become evident. Disparities are especially significant where free trade and the unrestricted movement of goods and services exist. The lack of uniformity and, in some cases, paucity of regulations can lead to public health risks and unfair competition. Future elimination of trade barriers calls for harmonization of safety requirements.

This chapter provides a summary of available information on rates of toxin loss by bivalve molluscs, a global overview of shellfish production and monitoring programmes, and a summary of available information on regulations governing paralytic (PSP), diarrhetic (DSP), amnesic (ASP) and neurotoxic (NSP) shellfish toxins and azaspiracids. Current activities in the European Union[1] (EU), Asia Pacific Economic Cooperation (APEC) area and in the USA to harmonize approaches to phycotoxin control are also described.

1. The European Union is currently made up of 15 countries: Austria, Belgium, Denmark, Finland, France, Germany, Greece, Ireland, Italy, Luxembourg, the Netherlands, Portugal, Spain, Sweden and the United Kingdom.

24.2 SHELLFISH PRODUCTION WORLDWIDE

The considerable decrease in wild fisheries catches has led to growth over the last decade in shellfish production worldwide and especially in the Asia–Pacific region. Countries of the Association of Southeast Asian Nations (ASEAN) are traditionally large consumers of seafood products, on which they rely heavily for subsistence and export income. Some of them have become world producers of several bivalve species. China is by far the main bivalve-culturing country and major world producer of scallops, mussels, clams and cockles, followed in terms of production by Japan, which ranks as the highest consumer of seafoods in the world, and the Republic of Korea (Table 24.1). Chinese Taipei, Indonesia, Malaysia and Thailand are also included among the major ASEAN producers. While almost the entire production of

TABLE 24.1 Production of molluscan shellfish in 1999 by major producing countries (metric tonnes)

	Mussels	Scallops	Clams and cockles	Oysters	Gastropods	Total
Australia	1.693	11.623	1.444	10.731	5.593	31.084
Canada	28.904	57.950	36.835	12.510	1.704	137.903
Chile	26.870	22.383	42.568	5.738	9.546	107.105
China	608.115	712.442	2.492.345	3.007.544	1.801	6.822.247
Denmark	96.215	0	246	8	1	96.470
France	64.621	15.741	11.206	137.161	8.758	237.487
Greece	32.772	0	16	49	0	32.837
Ireland	16.111	1.559	532	7.931	7.579	33.712
Italy	167.876	0	86.462	0	0	254.338
Japan	0	515.645	101.113	205.345	13.109	835.212
Korea, Rep. of	23.227	383	72.475	188.868	9.146	294.099
Malaysia	9.764	0	81.589	198	0	91.551
México	805	1.864	6.210	43.332	9.167	61.378
Netherlands	100.800	306	50.888	3.214	0	155.208
New Zealand	73.977	897	1.577	16.006	1.170	93.627
Peru	14.612	31.726	338	50	6.814	53.540
Philippines	15.498	62	282	13.793	282	29.917
Portugal	286	0	4479	653	196	5.614
South Africa	2.300	0	0	405	510	3.215
Spain	262.015	384	26.197	4.514	1.089	294.199
Thailand	61.800	0	87.133	22.917	0	171.850
Tunisia	8	0	48	0	0	56
Turkey	2.300	68	3.585	840	0	6.793
United Kingdom	17.507	25.137	14.387	1.566	6.261	64.858
United States	5.426	80.064	330.637	177.740	4.891	598.758
Venezuela	451	0	39.024	2.037	0	41.512

Source: Food and Agriculture Organization of the United Nations (FAO): http://apps.fao.org/fishery.

Japan is destined for domestic markets, some countries, such as the Republic of Korea, export a significant share of production. There is a general trend towards increasing export trade from ASEAN countries to Europe, Canada and the USA.

Among countries on the American continent, the USA leads in the production of bivalves, being ranked as the second-biggest world producer of clams and cockles and third-biggest producer of oysters and scallops. The USA is followed in importance by Canada which, like the USA, is seeking new products with more added value (Vieytes and Leira, 2000). The increase in recent years in Chile's production of scallops is noteworthy, as is the rise in clam and cockle production by Mexico and Venezuela. Mexico is the second-biggest American producer of oysters and Chile and Peru are the most important South American producers of mussels.

In Europe, the main molluscan shellfish producers are Spain, France, Italy, Denmark and the Netherlands. Total production in 1997 was about 1 million tonnes. Mussel production is of great importance in this area, with Spain, Italy and the Netherlands leading the production of cultured mussels and Denmark the harvest of wild mussels. In recent years, Greece has strongly increased its mussel catch and Ireland has strengthened its mussel industry, mainly destined for export markets. The markets for fresh and frozen mussels are almost exclusively in the EU. Italy leads clam production and France is the major producer of oysters. The Netherlands is the biggest producer of cockles and the United Kingdom the top producer of scallops and gastropods. Export activities are very important in the EU, although exchanges are mainly made between Member States. The Netherlands is by far the main mussel-exporting country, exporting mainly live mussels to neighbouring countries such as Belgium and Germany. Other exporting countries are Spain, Ireland, Germany and Denmark. Quantitatively, France, Belgium and Germany are the main importers, whereas the Netherlands tends to import in times of low production.

In the Pacific region, New Zealand and Australia are important producers of scallops and oysters. In recent years, the mussel production of New Zealand has experienced a sharp increase with almost the total production destined for export as frozen mussels (half-shell product) (data on shellfish production from FAO and Vieytes and Leira, 2000).

The data on shellfish production in Table 24.1 illustrate the importance of seafood in densely populated geographical areas strongly dependent on these resources for local consumption and where the exploitation of wild and cultured bivalves represents a significant part of their economic production. The impact of toxic phytoplankton on public health and the economy can be enormous in developing countries but also in developed countries which, although less vulnerable to phycotoxins in shellfish, have also suffered toxic outbreaks and enormous economic losses in fisheries and tourism.

24.3 Processes involved in harmful algal episodes

24.3.1 Ingestion and incorporation of toxic or noxious algae and substances

Some species of microalgae can produce harmful effects on shellfish populations either affecting them directly or by causing toxin accumulation that makes them toxic or undesirable to humans (reviewed in Blanco-Pérez, 2001). In some cases,

large blooms of microalgae modify substantially characteristics of the water such as pH, oxygen content, viscosity, etc., producing delays in shellfish growth, alterations of the typical shape or even mortalities. This alteration of the medium is sometimes accompanied by the production of substances that can be toxic for some shellfish species, increasing the probability of mortalities and leading to large economic losses. In some other cases the microalgae produce substances which are accumulated in the shellfish and which, without having a significant effect on them, make the organisms undesirable – by giving them a strange colour (e.g. the ciliate *Mesodinium rubrum*) or taste (e.g. diatoms of the genus *Rhizosolenia)* – or even toxic to humans.

Shellfish (bivalve molluscs, gastropods, crabs, lobsters and others) accumulate phycotoxins mostly by direct filtration of the algal cells or by feeding on contaminated organisms (e.g. carnivores and scavengers). The accumulation of a particular toxin is regulated by the balance of toxin intake and loss from/to the environment and by the transformations to/from other toxins. The toxicity of the molluscs depends on the concentration of the different toxins in the soft tissues – which in turn depends on the toxin burden and the weight of the tissues – and on the potency of each toxin. Rates of toxin accumulation of filter-feeding shellfish from toxic algae are toxin- and species-specific, both because the food ingestion of some mollusc species can be affected by the presence of toxic algae and because mollusc species differ substantially in their retention capability for each group of toxins. Some species of bivalve are known to reduce or completely suppress the toxic phytoplankton ingestion, in some cases to avoid toxic food or in other cases because of the toxic species' adverse effect on the mechanism for food capture (reviewed in Blanco-Pérez, 2001). Several PSP-producing species of the genus *Alexandrium* have been shown to produce both kinds of responses in several shellfish species (see Shumway and Cucci, 1987, Bricelj *et al.*, 1998, Lassus *et al.*, 1999 for PSP). One species of particular interest is the northern quahog or hard clam, *Mercenaria mercenaria* which, during an outbreak of a bloom of *Alexandrium tamarense* in Massachusetts (USA), remained innocuous when most bivalve species attained large toxin concentrations. Studies in our laboratory (Shumway) have shown that, in the presence of *A. tamarense*, the quahog first retracts its siphons then completely isolates itself from the external environment by means of shell valve closure. Efforts to induce toxicity by feeding *A. tamarense* were unsuccessful.

No effect on the feeding behaviour by DSP-producing species has been found to date, neither in natural episodes – usually produced by a very low concentration of *Dinophysis* species in the water – nor in the experimental intoxification of *Argopecten irradians* with *Prorocentrum lima* (Bauder *et al.*, 2001). *Pseudonitzschia multiseries*, which produces ASP toxins, had no effect on *Mytilus californianus* (Whyte *et al.*, 1995) but induced the valve closure in *Crassostrea gigas* (Jones, 1995). The same effect is produced by *Gymnodinium breve* (NSP) on *C. gigas* and *Braquiodontes recurvis* (Sievers, 1969).

The toxin contents and composition of the cells have been shown to have some effect, at least in PSP intoxification of *M. edulis* and *M. mercenaria* (Bricelj *et al.*, 1991).

As with the organic matter, not all the toxin contained in the cells is absorbed by the mollusc. The main variables that seem to control the process are the gut passage time (the time the food is maintained in the digestive system) and the digestibility and/or the postingestive selection of the toxic cells (Moroño *et al.*, 2001;

Bauder *et al.*, 2001). Toxic history and genetic differences also affect the incorporation of toxins into molluscs.

Toxicity of individual shellfish in any given area is highly variable (see White *et al.*, 1993; Chebib *et al.*, 1993, for PSP; Gilgan *et al.*, 1990; Douglas *et al.*, 1997; Arévalo *et al.*, 1998; Blanco *et al.*, 2002a, b, for ASP), probably mainly as a consequence of the also high inter-individual variability in the rates of food acquisition of most molluscs.

24.3.2 Detoxification

The rate of toxin loss is also toxin- and species-specific. The majority of available information concerns bivalve molluscs (see also Shumway, 1990 and Bricelj and Shumway, 1998); as these are the species most commonly reared in aquaculture, they are also the focus here. Table 24.2 summarizes selected data on toxin retention for a number of bivalve species.

Little is known about the factors that regulate the detoxification rate. Prakash *et al.* (1971) showed that PSP detoxification varied according to the season along the eastern coast of Canada; it is frequently assumed that low water temperatures retard toxin loss (see Shumway and Cembella 1993). Notwithstanding this, temperature seems to affect different toxins and species in different ways (no effect: PSP from *Saxidomus giganteus* (Madenwald 1985), *Mytilus galloprovincialis* (Blanco *et al.*, 1997); slower loss: DSP from *M. galloprovincialis* (Blanco *et al.*, 1999); faster loss: ASP from *Mytilus edulis* (Silvert and Subba Rao, 1993; Novaczek *et al.*, 1992), and *Pecten maximus* (Blanco *et al.*, in preparation).

Among the other factors studied to date, probably the amount of food is the most important one for PSP and DSP, increasing slightly the detoxification rate (Sampayo *et al.*, 1990; Marcaillou-Le Baut *et al.*, 1993; Blanco *et al.*, 1997, 1999). Metabolic processes in general seem to have little or no influence in regulating the loss of DSP and PSP toxins from mussels *M. galloprovincialis,* as no difference was found between groups with completely aerobic and partially anaerobic metabolisms (Moroño *et al.*, 1998). However, it seems that metabolic processes may affect domoic acid detoxification in *Pecten maximus* (Blanco *et al.*, unpublished data).

The rate of detoxification is highly dependent on the site of toxin storage within the animal. Toxins in the digestive gland are usually eliminated much more readily than toxins bound in other organs, with the noticeable exceptions of DSP toxins in *Argopecten irradians* (Bauder *et al.*, 2001) and domoic acid in *P. maximus* (Blanco *et al.*, 2002).

24.3.3 Toxin transformations

Most toxins undergo transformation in the organisms in which they accumulate, in some cases because the conditions (pH, redox potential, etc.) differ from the producer, in others because of the enzymatic activity of the accumulator. As toxin analogues usually differ in their toxic potency, the transformations produce a change in the whole toxicity of the animal without any input or loss of toxin. Many PSP toxins interconvert easily (see Bricelj and Shumway, 1998 for bivalves, Arakawa *et al.*, 1998 for crabs), producing increases or decreases in toxicity. A succession of derivatives of okadaic acid and dinophysistoxins 1 and 2 (e.g. diol esters, DTX4-like compounds) exhibiting different toxic potency can be present in the phytoplankton cells (for example) and, at least in some cases, can be reconverted to the parent

TABLE 24.2 Approximate times of toxin retention for various species of bivalve molluscs (time for toxin levels to fall below either quarantine or detection levels). Algal species are as given in original publications [1].

Species	Toxin source	Retention time	Reference
Ameghinomya antiqua	probably *Dinophysis acuta*	>6 months	Lembeye *et al.* (1993)
Anadara maculosa	*Pyrodinium bahamense*	6 weeks	Worth *et al.* (1975)
Argopecten irradians	*Prorocentrum lima*	>11 <60 days (viscera)	Bauder *et al.* (2001)
Arctica islandica	*Protogonyaulax tamarensis*	2 months *in vivo*	Shumway, unpublished
Aulacomya ater	probably *Dinophysis acuta*	6 months	Lembeye *et al.* (1993)
Choromytilus meridionalis	*Gonyaulax catenella*	3 months	Popkiss *et al.* (1979)
Clinocardium nuttalli	*Gonyaulax acatenella*	9 weeks	Quayle (1965)
Crassostrea cucullata	not specified, probably *Pyrodinium bahamense*	2 months	Karunasagar *et al.* (1984)
Crassostrea echinata	*Pyrodinium bahamense*	3 weeks in closed system; longer periods *in vivo*	Maclean (1975)
		4 months	Worth *et al.* (1975)
Crassostrea gigas	*Gonyaulax acatenella*	1–9 weeks	Quayle (1965; 1969); Sharpe (1981)
		1 month	Sribhibhadh (1963)
Crassostrea iridescens	*Gymnodinium catenatum*	>1 month	Mee *et al.* (1986)
Crassostrea virginica	*Gymnodinium breve*	2–6 weeks	Morton & Burklew (1969)
Meretrix casta	not specified, probably *Pyrodinium bahamense*	1 month	Karunasagar *et al.* (1984)
Mercenaria mercenaria	*Alexandrium tamarense*	2.1–3.6 weeks	Bricelj *et al.* (1991)
Modiolus auriculatus	*Pyrodinium bahamense*	6 weeks	Worth *et al.* (1975)
Modiolus modiolus	*Gonyaulax tamarensis*	up to 60 days [2]	Gilfillan *et al.* (1976)
Mya arenaria	*Gonyaulax acatenella*	5 weeks	Quayle (1965)
	Gonyaulax tamarensis	4–6 weeks	Prakash *et al.* (1971); Bicknell & Collins (1973)
		up to 45 days [2]	Gilfillan *et al.* (1976)
Mytilus californianus	*Gonyaulax catenella*	<1 month	Sommer & Meyer (1937) Sharpe (1981)
Mytilus edulis	*Protogonyaulax tamarensis*	10 days–7 weeks	Oshima *et al.* (1982);
		up to 50 days	Gilfillan *et al.* (1976) Prakash *et al.* (1971)
	Gonyaulax acatenella	11 weeks	Quayle (1965)
		4 weeks	Sharpe (1981)
	Gonyaulax excavata	2–3 weeks	Gaard & Poulsen (1988)

…/…

TABLE 24.2 *(Suite)*

Species	Toxin source	Retention time	Reference
	Dinophysis spp.	1 week	Haamer *et al.* (1989)
	Dinophysis spp.	8 weeks	Marcaillou-le Baut *et al.* (1990)
	Dinophysis spp.	8>42 days [2]	Marcaillou-le Baut *et al.* (1993)
	Dinophysis spp.	>10 months	Sechet *et al.* (1990)
	Dinophysis spp. (?) *Prorocentrum* spp. (?)	10 days	Quilliam *et al.* (1993)
	probably *Dinophysis acuta*	6 months	Lembeye *et al.* (1993)
Ostrea edulis	*Alexandrium tamarense*	>6 weeks	Shumway *et al.* (1990)
Patinopecten yessoensis	*Protogonyaulax tamarensis*	6 weeks–5 months	Oshima *et al.* (1982); Iioka *et al.* (1964)
*Perna canaliculus**	*Nitzschia pungens* f. *multiseries*	2 days	Mackenzie *et al.* (1993)
Placopecten magellanicus	*Protogonyaulax tamarensis*	6 month in closed system; can be toxic year round *in vivo*	Bourne (1965); Shumway *et al.* (1988)
	P. Pungens f. multiseries	>2 weeks (viscera)	Van Apeldoorn *et al.* (1999)
Protothaca staminea	*Protogonyaulax acatenella*	5 weeks	Quayle (1965)
Saxidomus giganteus	*Protogonyaulax acatenella*	2 years +	Quayle (1965); Anonymous (1974)
Saxidomus solidissima	*Gonyaulax catenella*	3 months–2.5 years	Quayle (1969)
Siliqua patula	*Pseudonitzschia* spp. (?)	>2 years	Wekell *et al.* (1993); Drum *et al.* (1993); Horner *et al.* (1993)
Spisula solidissima	*Alexandrium tamarense*	3 months-3+ years	Shumway *et al.* (1994; unpublished)
Spondylus sp.	*Pyrodinium bahamense*	still highly toxic after months	Worth *et al.* (1975)
Tresus capax	*Gonyaulax acatenella*	11 weeks	Quayle (1965)
Venerupis japonica	*Gonyaulax acatenella*	5 weeks	Quayle (1965)

1. Note : *Gonyaulax* and *Protogonyaulax = Alexandrium; Nitzschia = Pseudo-nitzschia.*
2. Dependent on initial level of toxicity.

* Laboratory study only; toxic organisms not identified in natural habitat.

toxins by the enzymes (during or after digestion) of the accumulator, very likely inducing significant changes in its toxicity. Simultaneously, OA, DTX1 and DTX2 can be converted to the corresponding acyl-derivatives (DTX3) (Fernández *et al.*, 1998; Moroño *et al.*, in press). ASP toxins have been less studied with regard to this aspect. Notwithstanding this, a number of DA isomers that bind to the kainate receptor less strongly (see Wright and Quilliam, 1995) have been described; consequently, the situation may be the same as that reported for PSP and DSP toxins.

24.3.4 Changes in shellfish biomass

The toxicity of an organism depends not only on the toxic burden and toxic potency but also on the amount of biomass in which the toxins are distributed. Any alteration of the weight of soft tissues which is not paralleled by an equivalent modification in the toxic burden produces changes in the toxicity per unit of biomass. For PSP- and DSP-contaminated shellfish, weight is not lost at the same rate as the toxins). In such cases, when the weight decreases, the same amount of toxin is concentrated in a smaller biomass and the toxicity per unit of biomass of the organism increases. On the other hand, when the organism increases its weight, the toxicity per unit of biomass decreases because the toxins are 'diluted' in a larger biomass. These situations have been found in several studies both in natural and laboratory conditions (see Bricelj and Cembella, 1995; Blanco *et al.*, 1997, 1999). With some combinations of species and toxins, the situation may differ from that already described. Thus, the loss of domoic acid from the king scallop seems related to loss of weight (Blanco *et al.*, 2002) and consequently the toxicity per unit of biomass is relatively independent from weight changes.

24.3.5 Toxin retention time

Toxin retention time by shellfish depends on the mechanisms analysed above; they are, therefore, species- and toxin-specific (Table 24.2). The differences in retention time are considerable. The mussels *Mytilus galloprovincialis* and *M. edulis* retain domoic acid for hours or days (Novaczek *et al.*, 1992; Blanco *et al.*, 2002a), whereas the scallop *Pecten maximus* (Blanco *et al.*, 2002b) retains this toxin for more than two years with a behaviour similar to that of *Saxidomus giganteus* and *Spisula solidissima* with PSP toxins (Quayle, 1965; Blogoslawski and Stewart, 1978; Chambers and Magnusson, 1950; Cembella and Todd, 1993; Shumway *et al.*, 1994).

Few data are available on the retention times of toxins by crabs and carnivorous gastropods. The general trend for PSP toxins appears to be towards long-term retention (Desbiens and Cembella, 1993; Shumway *et al*., 1995; Shumway, unpublished data) but ASP can be eliminated from dungeness crab Cancer magister at a relatively fast rate (Lund *et al*., 1997).

Generalizations regarding the uptake and retention of phycotoxins by shellfish should be avoided and the species- and toxin-specific characteristics should be taken into account before choosing a species for rearing in areas prone to toxic algal blooms, as well as when designing monitoring and sampling regimes.

24.4 Strategies to avoid intoxication and intoxification of shellfish

The exposure of shellfish to microalgal blooms can be reduced mainly by two procedures: reducing the algal biomass bloom or changing the location of the shellfish population. So far, the only strategy which has been proved efficient against the development of an algal bloom was the dispersion of clay over a proliferation of *Cochlodinium polykrikoides* in the Republic of Korea (Kim *et al*., 1998), but it presents the drawback of an obvious environmental impact. Other strategies (reviewed in Blanco-Pérez 2001 and partially in Elbrachter and Schnepff, 1998), such as the use of ultrasounds or chlorine to kill the algal cells or the use of several

viral, bacterial, fungal or protozoan parasites that lyse cells, have been experimented with but not used in real blooms. The second possible way of actuation consists in relocating the shellfish population (specially if they are cultured) or restricting the culture to places or depths at which the concentration of the harmful population is minimal (Desbiens and Cembella, 1993; Lodeiros *et al*., 1998; Franzosini *et al*., 2000).

24.5 Strategies to eliminate toxins from shellfish

Two groups of techniques are being used to eliminate toxins from the mollusc. The aim of the first group is to obtain a live non-toxic mollusc and includes techniques such as temperature or salinity stress, ozone or chlorine treatments, transplantation, etc. that accelerate the loss of toxins from the organisms or, at least, prevent reintoxication. The aim of the second group is to obtain products free of toxins after applying specific processing procedures.

24.5.1 Strategies to obtain living shellfish free of toxins

Various attempts have been made to detoxify live shellfish contaminated with paralytic shellfish poisons to reduce the duration of 'off-market' times. The most obvious method is to transfer shellfish to waters free of the toxic organisms which also have environmental conditions favouring depuration. Unfortunately, the small effect of the environmental conditions on the PSP- and DSP-detoxification rate of the mussel Mytilus galloprovincialis (Blanco *et al*., 1997, 1999) makes transplantation more useful to prevent reintoxication than to increase the depuration rate. Notwithstanding this, Blanco *et al*. (manuscript in preparation) recently found that the king scallop *Pecten maximus* depurates domoic acid much faster when suspended from rafts than in natural conditions. Transplantation is being used in several countries to benefit from these two methods. Detoxification of PSP toxins using temperature or salinity stress has also been tried with marginal success (Gilfillan *et al*., 1976; Blogoslawski and Neve, 1979). Instantaneous electric shock treatment accelerated toxin excretion in scallops (Kodama *et al*., 1989). Reduced pH has been tried as a means of detoxifying butter clams but without success (Anonymous, 1966; Neal, 1967). Chlorination has been used in France; however, this process alters the flavour of the shellfish and thus decreases marketability.

Ozone has been touted as an effective means of reducing toxicity although its usefulness is questionable. Several early studies reported ozone to be effective in inactivating PSP toxins in shellfish exposed to *Alexandrium tamarense*, *A. catenella* and *Karenia brevis* blooms (Thurberg, 1975; Blogoslawski *et al.*, 1975, 1979; Dawson *et al.*, 1976; Blogoslawski and Stewart, 1978). Blogoslawski *et al.* (1973) also suggested that ozone could be used to inactivate *Karenia (Gymnodinium) brevis* toxins. More recently, preliminary studies by Gacutan *et al.* (1984, 1985) demonstrated that both ozone gas and PVP-iodide-iodine may effectively inactivate PSP toxins from *Perna viridis* contaminated by *Pyrodinium bahamense*. However, a subsequent study by White *et al.* (1985) produced results contradictory to previous studies in that no detoxification occurred in *Mya arenaria* exposed to ozone treatments. In a review (Blogoslawski, 1988), it was again suggested that ozonized sea water could be of value in detoxification of shellfish contaminated recently by the vegetative cells of toxic (PSP) dinoflagellates. In a study during a red-tide outbreak,

it was shown that ozone treatment of the sea water did prevent shellfish (*Mytilus edulis, Mya arenaria* and *Geukensia demissa*) from accumulating paralytic shellfish poison. Blogoslawski concluded that inactivation could be achieved in bivalves exposed to and contaminated by motile PSP dinoflagellate cells without measurably altering the physical state of the treated bivalves and that this inactivation could be achieved in a marketable species such as *Mya* within an economically feasible time-frame (Blogoslawski *et al.*, 1979). Most recently, Fletcher *et al.* (1998) concluded that ozone was ineffective in reducing toxins from *K. brevis* in the oyster *C. gigas*. Ozone is useless in detoxifying cysts or in bivalves that have ingested cysts or have the toxins bound in their tissue over long periods. Furthermore, detoxification of algal toxins, especially paralytic shellfish poisons, over long periods is not economically feasible. We do not recommend ozone as a practical or safe means of eliminating algal toxins from shellfish. At present, the economic feasibility of efficiently detoxifying shellfish on a large scale in artificial systems is not promising, perhaps with the exception of the oyster *Crassostrea gigas* contaminated with PSP toxins, which responds to an increased food supply with a substantial increment in its rate of toxin loss (Lassus, personal communication) and has considerable commercial value. In areas prone to regular outbreaks of toxic algal species, aquaculturists and commercial fishermen alike must still depend on reliable monitoring systems to warn of toxic shellfish and plan their activities accordingly. Through the combined efforts of an intensive monitoring programme and the culture of 'rapid release' species (e.g. *Mytilus edulis*), bivalves known to avoid toxic dinoflagellates (e.g. *Mercenaria*, most oysters) or scallops (adductor muscles are rarely if ever toxic), economic losses can be kept to a minimum (see also Shumway *et al.*, 1988).

24.5.2. Strategies to obtain shellfishery products free of toxins

The second group of techniques ranges from the simple process of selective evisceration to cooking or more complex industrial processing techniques. Some species accumulate toxins preferentially in one or in a few organs. In these cases, an easy way to eliminate most of the toxin is to remove such organ(s) (evisceration). Obviously, this procedure can only be used when the species is big enough to allow the dissection of the toxic anatomical part and the species has enough commercial value to compensate the usually large economic cost of dissection. This procedure is currently being applied in the EU for the king scallop *Pecten maximus* contaminated with ASP toxins. Recent EU legislation (Decision 2002/226/EC) has established an exception to the general regulation for the exploitation of bivalve molluscs, in order to allow this procedure under specific conditions; it is proving useful in mitigating the economic impact of the very long detoxification time of this species. The same procedure could be used with other bivalve species such as the razor clam *Siliqua patula* with ASP toxins (Horner *et al.*, 1993), or *Argopecten irradians* with DSP toxins (Bauder *et al.*, 2001) but also with gasteropods, such as *Haliotis tuberculata* and *Haliotis midae*, that concentrate most of PSP in epipodial fringe (Bravo *et al.*, 1999, 2001; Pitcher *et al.*, 2001).

Very recently, the basis for a procedure to eliminate DSP toxins from molluscs by means of critical point extraction have been set up by González *et al.* (2002), pointing to an interesting approach to eliminate DSP toxins but also other lipophilic toxins. The technique requires dehydration of the product and therefore it will only be useful for products that require, or at least tolerate, this process.

Cooking has also been promoted as a possible means of detoxifying shellfish contaminated with paralytic shellfish poisons. Although cooking does not eliminate the danger of intoxication, it may effectively reduce toxicity to safe levels if initial levels of toxicity are low. Pan-frying seems to be more effective in reducing PSP levels than other methods of cooking (Medcof *et al.*, 1947; MacDonald, 1970). When clams or mussels are steamed or boiled, toxins lost from the tissues are contained in the cooking liquid rendering the fluids extremely toxic. Vieites *et al.* (1999) showed that more than 30% of the PSP toxins contained in mussels (*Mytilus galloprovincialis*) are transferred to the water after cooking them at 97°C for two minutes, whereas real destruction of the toxin was about 20%.

Commercial canning has been shown to reduce toxicity (paralytic shellfish poisons) of soft-shell clams, *Mya arenaria*, by as much as 90%. A toxicity level of 160 µg STXeq 100 g^{-1} for soft-shell clams and mussels (*Mytilus edulis*) to be canned was established in the 1950s in Atlantic Canada and remains in effect today (Cembella and Todd, 1993). Noguchi *et al.* (1980) (see also Nagashima *et al.*, 1991) showed that toxicity levels of PSP-infested scallops could be reduced during canning processes. They demonstrated that, during retorting (110°C, 80 min, or 122°C, 22 min), most of the PSP toxins could be eliminated (maximum initial level 102 MU g^{-1} digestive gland), whereas heating (70°C, 20 minutes) followed by washing was less effective in reducing the toxicity below the quarantine limit of 4 MU g^{-1}. Recent efforts in Spain (Berenguer *et al.*, 1993) have demonstrated that toxicity levels of Mediterranean cockles (*Acanthocardia tuberculatum*) may be significantly reduced via canning. Total toxicity of cockles (initial levels of approximately 800 g STXeq 100 g^{-1}) was reduced to <35 g STXeq 100 g^{-1} after cooking. These authors demonstrated that the decreases in PSP toxicity obtained by commercial processing were sufficient to warrant canning as a practical means of obtaining a legal and acceptable product when the toxin levels were moderate. Similar attempts have been made to detoxify surfclams (*Spisula solidissima*) via canning but results to date are inconclusive. During the canning process of PSP-contaminated *Mytilus galloprovincialis*, about 30% of the toxin goes to the packing liquid and about 33% is destroyed (when packaged in brine) (Vieites *et al.*, 1999). It has also been shown that the thermal treatments associated with canning induce transformations between the different PSP toxins, with the consequent changes in toxicity (Vieites *et al.*, 1999; Indrasena and Gill, 1999). The effectiveness of canning as a means of reducing PSP toxicity levels below quarantine levels seems to be dependent on the initial profile of toxins and, in some cases, on the initial level of toxicity and should be approached with great caution.

Domoic acid was shown to decrease from the scallop tissues using freezing followed by canning (Leira *et al.*, 1998) but it seems that only the freezing step reduced the overall toxin content of the product.

With the exception of the study by Berenguer *et al.* (1993), no useful methods have been devised for effectively reducing phycotoxins in contaminated live shellfish. All methods tested to date have been unsafe, too slow, economically unfeasible or have yielded products unacceptable in appearance and taste. Given the global increase in harmful algal blooms and the continually growing interest in the culture of bivalve molluscs, further efforts are needed to develop effective means of detoxifying shellfish contaminated with phycotoxins. Failing the development of any such methods, greater efforts will need to be expended to monitor shellfish for the presence of phycotoxins.

24.6 Strategies to minimize the risk of toxic shellfish to human health

The strategies to minimize the risk for human health are based on the definition of allowable toxin levels in seafoods and in the determination of those levels in the exploited shellfish populations, which includes the use of suitable methodology and the establishment of adequate monitoring programmes.

24.6.1 Allowable toxin levels and methods: the basis for regulation

Regulations on action levels should ideally be based on a risk assessment that comprises hazard identification (which involves identification of the toxins causing adverse health effects), hazard characterization (which implies evaluation of the adverse health effects associated with the presence of the toxins in seafoods), exposure assessment related to the evaluation of the likely intake of phycotoxins and risk characterization (which involves quantitative estimations of the probability of adverse health effects in a given population) (Aune, 2001).

Epidemiological data and toxin kinetics studies, action mechanism, target organs and dose-response relationships are required for the risk assessment process. In addition, the capability of testing procedures to identify and quantify toxin levels in food should be taken into account when establishing regulations. In the case of some marine toxins, especially some polyether toxins, there is a lack of such studies; the action levels have been set on the basis of sparse toxicity data mainly originating from human poisoning episodes, the detection limits of the available methods or following the specifications of other countries. Robust toxicological studies are lengthy and expensive to conduct and involve the use of large amounts of pure toxins which are not available in most cases.

Accurate methods of quantification have to be available because legislation calls for means of control. However, the development and validation of suitable testing methods has also been hampered by the lack of pure toxins and certified reference materials (Van Egmond *et al*., 1992). Most countries still rely on animal bioassays to detect PSP and DSP but the ethical aspects of these tests have led to growing resistance from animal welfare groups. It should also be borne in mind that it is unrealistic to establish an action level lower than the actual limit of detection even though this might be desirable from the toxicity point of view. The elucidation of new toxins, as well as the wide range of seafood that can act as toxin vectors, make the establishment of rational guidelines and regulations more difficult. Despite the dilemmas, a number of countries have established limits and regulations for phycotoxins in shellfish.

Paralytic shellfish poisoning (PSP)

Of all seafood poisonings, PSP represents one of the most severe threats to public health, affecting most coastal areas in the world. Saxitoxin (STX) and analogues are mainly produced by a number of dinoflagellates belonging to the genera *Alexandrium*, *Gymnodinium* and *Pyrodinium* (Yasumoto and Murata, 1993). Although bivalve molluscs are the main PSP vectors, gastropods, crustaceans and pelagic fish have also been reported as vectors of these toxins (Shumway, 1995). Many affected countries have established regulations including methods and action limits. Table 24.3 shows countries known to have PSP regulations in force.

TABLE 24.3 Regulations for paralytic shellfish poisons in various countries

Country	Product	Toxin(s)	Tolerable level[a, b]		Method of analysis	Remarks
Australia			80 µg 100 g^{-1}	-	Mouse bioassay	
Canada	Molluscs	PSP	80 µg 100 g^{-1}		Mouse bioassay	Products having levels between 80–160 µg 100 g^{-1} may be canned
European Union[c]	Bivalve molluscs	PSP	80 µg 100 g^{-1}	V	(Mouse) bioassay in association if necessary with a chemical detection method	If the results are challenged, the reference method is the biological one
Guatemala	Molluscs	Saxitoxin	400 MU 100 g^{-1}		Mouse bioassay	
Hong Kong China	Shellfish	PSP	400 MU 100 g^{-1}		Mouse bioassay	
Japan	Bivalves	PSP	400 MU 100 g^{-1}		Mouse bioassay	
Korea, Rep. of	Bivalves	Gonyautoxins	400 MU 100 g^{-1}		Mouse bioassay	
New Zealand	Shellfish	PSP	180 µg 100 g^{-1}		Mouse bioassay	
Norway	All types of mussel	PSP	180 µg 100 g^{-1}		Mouse bioassay	
Panama	Bivalves	PSP	400 MU 100 g^{-1}		Mouse bioassay	
Philippines			40 µg 100 g^{-1}			
Singapore	Bivalves	Saxitoxin	80 µg 100 g^{-1}		Mouse bioassay	
United States	Bivalves	PSP	80 µg 100 g^{-1}		Mouse bioassay	ISSC co-ordinates the Shellfish Programs administered by individual states
Uruguay	Bivalves	PSP	80 µg 100 g^{-1}		Mouse bioassay	
Venezuela	Bivalves	PSP	80 µg 100 g^{-1}		Mouse bioassay	

a. For consistency of presentation, all action levels are expressed as MU 100 g^{-1} or µg 100 g^{-1}.
b. MU: mouse unit.
c. European Union countries: Austria, Belgium, Denmark, Finland, France, Germany, Greece, Ireland, Italy, Luxembourg, the Netherlands, Portugal, Spain, Sweden and the United Kingdom.
Source: Van Egmond *et al.*, 1992, Andersen, 1996).

The main method used for regulatory purposes is the mouse bioassay (AOAC, 1990), which has been validated and standardized by the Association of Official Analytical Chemists (AOAC). This reference method is the only procedure recognized internationally for quantifying PSP toxicity, although with some variations in the acceptable regulatory level and in the units used for expression of the toxicity: 40 µg PSP 100 g^{-1}, 80 µg PSP 100 g^{-1} or 200 MU 100 g^{-1}, 400 MU 100 g^{-1} (Van Egmond *et al*., 1992). Most countries have agreed on 80 µg saxitoxin (STX) equivalent 100 g^{-1} shellfish meat.

The effectiveness of some procedures (evisceration, canning, etc.) in reducing PSP toxicity has led some countries to establish different limits depending on the form of consumption of the shellfish. Thus, in Canada and the USA, shellfish destined for canning or subjected to an evisceration step might be harvested with PSP toxin amounts higher than 80 µg 100 g^{-1} (Cembella and Todd, 1993; NSSP, 1990). In the case of EU legislation, the application of detoxification procedures is limited by Council Directive 91/492/EEC that prohibits the harvesting of toxic shellfish. There is only one exception (in the case of PSP) and this applies to the species Acantochardia tuberculata destined for canning, allowing Spain to authorize harvesting when PSP levels in the edible parts exceed 80 µg STXeq per 100 g tissue but are less than 300 µg STXeq per 100 g tissue (Decision 96/77/EC). The bivalves must undergo a heat treatment clearly defined in the Annex of the EU Decision, the final product must not contain a PSP toxicity level detectable by the mouse bioassay, and each lot must be tested (Burdaspal *et al*., 1998).

Diarrhetic shellfish poisoning (DSP)

Among all the groups of marine toxins, those included in the DSP group are the subject of greatest controversy. Three groups of toxins have historically been included in the DSP group: okadaic acid (OA) and dinophysitoxins (DTXs) produced by Dinophysis and Prorocentrum species, pectenotoxins (PTXs) produced by some Dinophysis species and yessotoxins (YTXs), produced by Gonyaulax grindleyi and Lingulodinium polyedra (Yasumoto *et al*., 1989; Yasumoto and Murata, 1990; Satake *et al*., 1998; Tubaro *et al*., 1998). These bioactive compounds share polyether cyclic structure and solubility properties; neither extraction procedures nor conventional mouse bioassays discriminate between them. However, their biological activity and toxicological properties are significantly different. There is no general consensus as to which liposoluble toxins should be regarded as DSP toxins, which ones should be monitored and regulated and which are the most appropriate testing procedures and acceptable levels.

Concerning sanitary control of DSP toxins, there are disparities in methods and in the criteria for positive results. Mammalian bioassays are widely applied for DSP toxicity determination. However, there are great differences in the procedures' performance. Assay selectivity, specificity and toxin recovery depend greatly on the selection and ratio of the organic solvents used for extraction. Bioassay procedures as diverse as the oral dosage rat bioassay (Kat, 1983) and the intra-peritoneal injection mouse assay are not equivalent because the former technique quantifies only the diarrhetic effect of certain DSP toxins, whereas the latter assay provides an estimate of total DSP toxicity (Cembella *et al*., 1995).

Regarding regulatory levels, most countries have set the limit at the detection limit of the analytical method used. The first country to establish a limit was Japan – 5 MU 100 g^{-1} shellfish tissue, based on an epidemiological study (Yasumoto *et al*.,

1978). This limit has also been established in the Republic of Korea and New Zealand. In practice, most countries applying mouse bioassays use survival time for the determination of the toxicity but without a consensus on the appropriate observation period; the acceptable criteria can vary between 'two out of three mouse deaths in less than five hours' to 'two out of three mouse deaths in less than 24 hours'.

Chile, Thailand, Turkey, Uruguay and Venezuela regulate DSP on the basis of the Yasumoto mouse bioassay (1978) with a survival time of 24 hours as the criterion for a positive result. Canada and the USA have no official regulations on DSP, although Canada may issue informal advice on positive results by mouse assay and/or the fluorescence high-performance liquid chromatography (HPLC) method.

Regarding DSP control in the European Union, the EU Directive 91/492 established that the customary biological method must not give a positive result for the presence of DSP in the edible part of the molluscs but it did not clarify the interpretation of a positive result and which biological methods should be used. Important progress has been made recently with the approval of Decision 2002/225/EC laying down detailed rules for implementation of the Directive 91/492 as regards the maximum levels and the methods of quantification of OA and Dinophysistoxins, Pectenotoxins, Yessotoxins and Azaspiracids in bivalve molluscs, echinoderms, tunicates, and marine gastropods. Regarding maximum levels, the following has been stated: A) The maximum level of okadaic acid, dinophysistoxins, and pectenotoxins, together shall be 160 µg of okadaic acid equivalents kg^{-1} edible part (the whole body or any part edible separately). B) The maximum level of yessotoxins shall be 1 mg of yessotoxin equivalents kg^{-1} edible part (the whole body or any part edible separately). C) The maximum level of azaspiracids shall be 160 µg of azaspiracid equivalents kg^{-1} edible part (the whole body or any part edible separately).

Concerning detection methods, the new Decision states that, in addition to biological testing methods, alternative detection methods such as chemical methods and in vitro assays should be accepted if it is demonstrated that the performance of the chosen method is not less effective than the performance of the biological method and that their implementation provides an equivalent level of public health protection. The compounds to be detected are: OA and Dinophysistoxins, PTX1 and PTX2, YTX, 45 OHYTX, homo YTX and 45 OH homo YTX AZA1, AZA2 and AZA 3. However, if new analogues of public health significance are discovered, they should be included in the determinations. Standards have to be available before chemical analysis becomes possible and total toxicity will be calculated using conversion factors based on the toxicity data available for each toxin. The performance of these methods should be defined after validation following an internationally agreed protocol. Concerning biological methods, it is stated that a suite of mouse bioassay procedures differing in the test portion and in the solvents used for the extraction and purification steps can be used but that sensitivity and selectivity depend on the choice of the solvents and that this should be taken into account when making a decision on the method to be used, in order to cover the full range of toxins. With regard to the mouse bioassay, it has been established that the death of two out of three mice within 24 hours after inoculation into each of them of an extract equivalent to 5 g of hepatopancreas, or 25 g of whole body, should be considered a positive result for the presence of one or more of the toxins of the groups regulated in the Decision at levels above those allowed.

Although the full implementation of this new Decision on the basis of chemical methods and/or in vitro assays still requires a big effort to develop toxin standards,

toxicological studies and validation of alternative methods, it represents an important step towards harmonizing phycotoxin control approaches.

Amnesic shellfish poisoning (ASP)

ASP is produced by the ingestion of seafood contaminated with domoic acid (DA), an excitatory amino-acid produced by some species of the pennate diatom genus Pseudo-nitzschia. The first toxic outbreak took place in Canada in 1987 and toxic episodes, although not human poisonings, have been reported in other regions. Countries that have established regulations for ASP are Canada, the USA, New Zealand (Trusewich *et al*., 1995; Wright *et al*., 1989; NSSP, 1990), Chile, Peru, and European Union Member States (EU Directive 91/492/EEC amended by Directive 97/61/EC). The limit is 20 mg kg^{-1} edible meat. The US Federal Food and Drug Administration (FDA) has set a quarantine level of 30 ppm of DA in cooked viscera of dungeness crabs (Shumway, 1995). HPLC methods have been established for regulatory purposes.

In order to minimize the economic impact produced by the slow ASP detoxification process of certain pectinidae, the EU has recently approved Decision 2002/226/EC allowing the harvesting of Pecten maximus and Pecten jacobeous with DA levels higher than 20 mg kg^{-1} and lower than 250 mg kg^{-1} if they are destined to be placed on the market after total removal of hepatoancreas, soft tissues or any other toxic contaminated part. On the basis of this Decision, a restricted harvesting regime of scallops with DA concentration (whole body) >20 mg g^{-1} and intended for processing can be initiated if DA concentration in the whole scallop (whole body) is <250 mg^{-1} and if the DA concentration of the anatomical parts intended to be placed on the market and analysed separately, (edible parts) is <4.6 mg^{-1} DA. The latter condition was introduced to take into account the large inter-animal variability existing in the concentration of DA in scallop organs. It is not a lowering of the regulatory limit. It assists in ensuring that the chance of the DA concentration in the scallop edible part exceeding 20 µg g^{-1} is less than 1 in 1,000 upon harvesting (probability "p" of 0.1%). It is based on the analysis of 10 pooled individual edible parts and a 50% coefficient of variation (as a measure of inter-animal variability in DA concentrations).

In addition to the conditions above for allowing harvesting, additional requirements concerning sampling, number of individuals to make up the analytical sample, transport conditions, authorized establishments, testing of the end product and traceability measures have been also established in the EU Decision.

Neurotoxic shellfish poisoning (NSP)

NSP is a seafood intoxication produced after the ingestion of shellfish contaminated with brevetoxins produced by the dinoflagellate *Karenia (Gymnodinium) brevis* and related species. The USA and New Zealand are currently the main areas affected directly by NSP (MacFarren *et al*., 1965; Ishida *et al*., 1995). The testing procedure in use in the USA is the mouse bioassay (APHA, 1985) and the acceptable level is 20 MU 100 g^{-1}. In New Zealand, a mouse bioassay equivalent to the APHA method (Hannah *et al*., 1995) is in use and 20 MU 100 g^{-1} is also employed as an acceptable level (Trusewich *et al*., 1995). The EU Directive makes no mention of NSP toxins as they have not been reported to occur at significant levels in European coastal waters.

Azaspiracid shellfish poisoning (AZP)

Azaspiracids Poisoning (Ofuji *et al*., 1999) is caused by the ingestion of shellfish contaminated with Azaspiracids, a novel group of polyether toxins recently identified in shellfish from Ireland, Norway and the United Kingdom (James *et al*., 2002) and reponsible for several outbreaks. AZP symptoms are similar to those of DSP; they include nausea, vomiting, severe diarrhoea and stomach cramps. *Protoperidinium crassipes* has been identified as an algal source of AZAs (Yasumoto, unpublished data). Recently, the control of AZAs in shellfish has been regulated by EU Decision 2002/225/EC establishing a maximum level of AZAs of 160 μg kg^{-1} edible part. In contrast with other lipophilic toxins, such as okadaic acid and analogues that mainly remain within the hepatopancreas, azaspiracids can be accumulated in whole shellfish tissues. In addition to LC-MS methods, a modification of the Yasumoto mouse bioassay (Yasumoto *et al*., 1984) involving the analysis of whole shellfish tissues is suitable for detecting AZAs in shellfish at the levels established in the aforementioned Decision (Yasumoto, manuscript in preparation).

24.6.2 Monitoring programmes: putting regulations into practice

Once having established a regulatory basis, a large number of countries worldwide have established phycotoxin monitoring programmes to prevent risks for public health and minimize economic losses for aquaculture and fisheries. The basic components of a programme – the authorities responsible for its application, the involvement of the industry and the organization of the inspection services – very much depend on the country or geographical area. In many cases, the programme includes surveillance for potential toxic algal species (identification and quantification) and monitoring of toxin content in shellfish by means of a range of testing procedures. Results are compared with allowed levels established in the regulations to prevent unsafe shellfish being placed on the market. In addition, some countries complement phytoplankton and toxicity data with the monitoring of biological, chemical and physical parameters such as water temperature, salinity, nutrients, chlorophyll, water stratification, current circulation, etc. This information, together with the weather and oceanographic conditions, constitutes a valuable prediction tool. Information on water-mass distribution and movements obtained from satellite imagery is also used to predict the appearance of phytoplankton populations. The application of airborne spectrographic sensing technologies and information provided by moored installations of ocean optical equipment have been proposed as effective monitoring tools.

Whatever the features of the programme, an algal and shellfish sampling protocol over time and space is designed; this includes defining of an adequate location and the number of sampling sites; there must also be sufficient sampling frequency to document spatio-temporal changes in phytoplankton and toxins in shellfish and to cover the risks of rapid rises in shellfish toxicity. Taking into account the changing nature of phytoplankton populations, sampling should be carried out at least weekly (Andersen, 1996), intensifying during the development of a toxic episode.

The programme is usually designed on the basis of oceanographic information, historical data on phytoplankton occurrence in the area, local needs, harvesting periods, the knowledge of the detoxification kinetics of the species under control, etc. Algal and shellfish sampling is usually performed by the authorities although, in some countries, local producers, industry and even volunteers may carry out sampling under the supervision of the authorities.

The economic importance of the resource and therefore the available funding, not to mention its spatial concentration, usually determines the features and extent of the programme, which may be mostly funded by government (e.g. Galician Rias in north-western Spain); by industry (e.g. in Denmark); or by both (e.g. in New Zealand). The more information is available on the spatio-temporal evolution of the phytoplankton populations in a given production area, the better the assessment of closure or opening of small zones over a short period. On the contrary, if the costs of regular monitoring are not affordable, production areas may be kept closed for prolonged periods when a risk of toxic episodes exists. In extreme cases of chronic bloom situations and no available funds, production areas may be permanently closed to avert loss of life, as in the case of some shellfish farms in ASEAN regions (Corrales and Maclean, 1995).

In addition to a suitable sampling programme, an adequate transport system for the samples should be developed, including suitable conditions for preserving these until they reach the laboratory. Once potentially toxic algal species have been identified and quantified, and shellfish tested using biological and chemical methods, results are evaluated and delivered to decision-makers. An extremely important issue is the time between sampling and the issuing of results, since this determines the efficacy and reliability of the control system. The elapsed time should be as short as possible (preferably no longer than one to two days), in order to decide whether or not to prohibit harvesting.

Information obtained from monitoring of toxic phytoplankton is used for different purposes: as an early warning tool, to set threshold levels for increasing sampling frequency and toxin testing in shellfish and even as a criterion for closing production areas when the number of potentially toxic algal cells exceeds certain critical concentrations. There is no general rule for defining a harmful concentration of cells; the criteria depend very much on the specific toxic species, ranging from hundreds (*Dinophysis* spp.) to millions (*Pseudo-nitzschia* spp.) of cells. However, there is not always a good correlation between cell counts and toxicity in shellfish. Significant inter- and intra-species variability in toxin profile and toxin content has been reported for many phytoplankton species even from the same area and over a short period. In addition, some shellfish species can store toxicity for long periods, so that toxicity remains many months after the toxic cells bloomed. ASP toxicity in scallops can remain even years after *Pseudo-nitzschia* blooms. Nor should the contribution of cysts to shellfish toxicity be underestimated.

Some countries rely on both cell counts and shellfish toxin content to decide on the closure of production areas, whereas others make the decision only on the basis of the toxicity in shellfish. Although information on both phytoplankton and toxin content is highly desirable, shellfish testing is more reliable from the point of view of protecting consumers.

The strategy used to monitor the Galician Rías, an area of intense aquaculture and consequently endowed with resources which are mostly concentrated in a relatively small area, illustrates how intensive a monitoring programme can be in time and space if the value of the resources is significant. The design of the programme was based on the fact that toxic events are possible at any time of year and that toxicity levels affect only small areas, whereas harvesting closures previously affected wider areas. To better predict the appearance, evolution and disappearance of toxic species, the production areas have been divided into small zones and subzones and the exploited species have been differentiated in terms of intoxication–

detoxification dynamics and harvesting schedules. Depending on factors such as oceanographic and weather conditions and the levels of toxic species found, various action plans including different sampling frequencies (from weekly to daily) have been established (Fig. 24.1). All data generated from the programme – oceanographic variables, phytoplankton composition, weather forecast, surface temperatures, toxin analysis – are processed using the same computer software, which updates the action plan twice a day. Any change in the plan is notified by fax and e-mail to all the organizations of the administration, industry and research centres. The number of sampling sites and frequency of sampling have increased, but the duration of quarantine periods and the extent of affected areas have been reduced considerably, allowing better management of the resources and minimizing economic impacts (Mariño *et al*., 1998).

A monitoring programme like the Galician one may be unfeasible, specially from the economic point of view, in other areas in which the resources are more dispersed and/or have less economic and social importance. Within the USA, the State of Maine has one of the most comprehensive monitoring programmes for paralytic shellfish poisons (see NSSP, 1990, Part 1, Section C, Appendix A). This programme was necessitated by yearly occurrences of toxic shellfish and has been used as a template for establishing monitoring programmes in many other regions worldwide. Its purpose is to ensure that only safe shellfish are harvested. Years of practical experience have afforded an opportunity to modify continually the sampling programme to reflect better increasing knowledge of potential toxic areas as well as the changing utilization of shellfish. Instead of 'primary-key stations', areas of similar toxic patterns are used. At the beginning of the PSP testing year, shellfish samples are collected from each of these areas to determine the background level of toxicity. Sampling stations from these areas are sampled each week from April to October, regardless of toxin patterns. When shellfish show any toxicity, sampling is expanded until stations of no toxicity are found. This sampling programme allows for closures to be made in a safe manner. Maine's law and regulations require the immediate closure of toxic shellfish harvest areas, and embargo or confiscation of all suspect shellfish. When necessary, licences and certificates may be suspended. Administrative actions are accomplished on a same-day basis.

Maine's PSP Monitoring Programme is continually modified to reflect increasing knowledge as to where toxic shellfish are likely to occur, as well as changing utilization of shellfish. The programme is carried out on a yearly basis from April until October. Based on areas of similar toxin patterns, the coast of Maine has been divided into 18 areas from south-west to north-east. At the beginning of the PSP testing year, shellfish samples (mussels, *Mytilus edulis*; clams, *Mya arenaria*) are collected weekly from 'primary' sampling stations from these areas. Early-season shellfish samples determine the background level of toxicity in an area (hopefully below the limit of detection sensitivity of the test). Whenever there is any rise in toxin in an area, the sampling of shellfish is increased to adjacent sampling stations so that when a closure is judged necessary adequate data are available to make a proper public health-oriented decision. Closures are made and safety zones put in place, frequently on the basis of previous PSP closures for that area. Shellfish are returned to the laboratory under refrigeration and tested as soon as possible under the standard mouse bioassay. Whenever toxin levels are found approaching the quarantine level of 80 µg STXeq per 100 g of shellfish, the area is closed to shellfish harvesting.

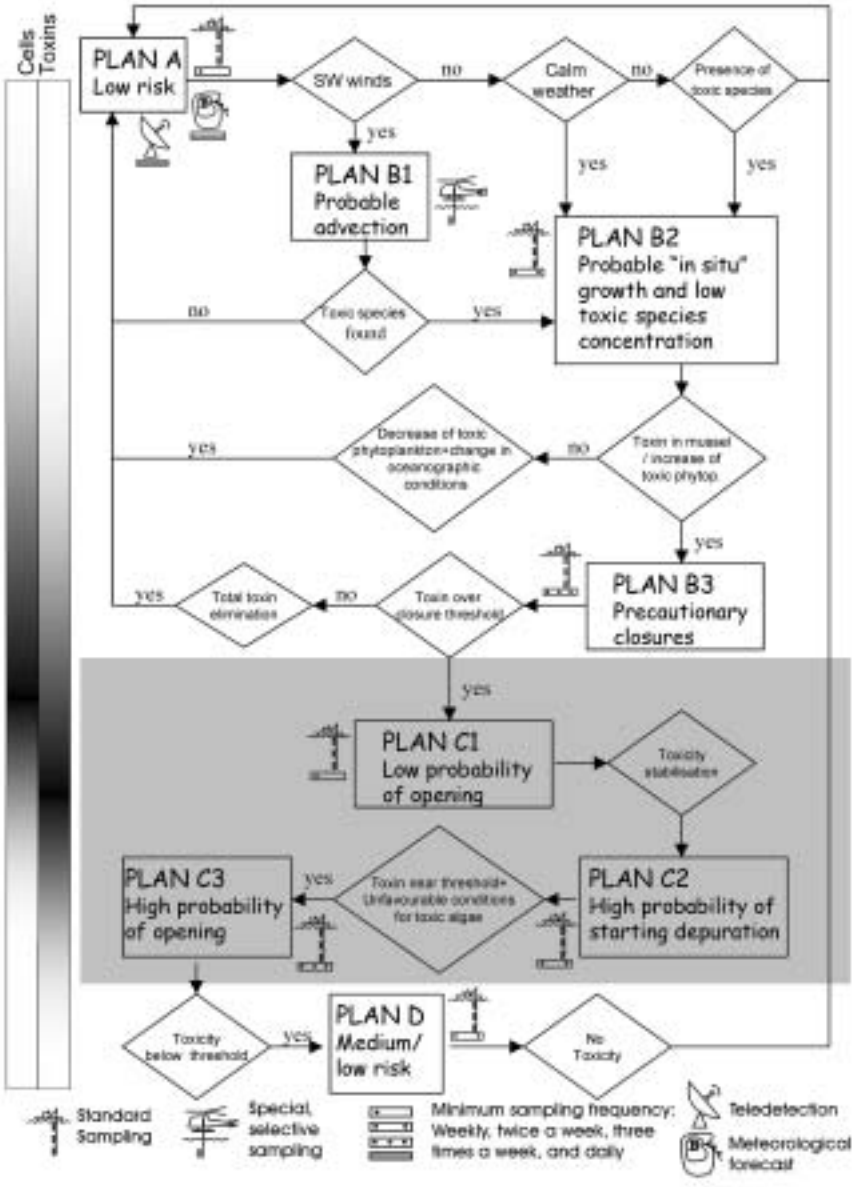

Figure 24.1
Action plans

Closures are made on a species basis if adequate information is available to demonstrate that not all species are toxic. Species closures require increased sampling of the 'toxin-free' shellfish. In areas where it can be justified, increased sampling will permit a partial opening of an area where non-toxicity is proven. Shellfish such as the ocean quahog, *Arctica islandica*, are sampled from contract fishing boats by department personnel. Areas where quahogs and similar species cannot be sampled are closed because they cannot be regarded as safe without sampling. Reopening of closed areas is dependent on continued toxin levels of less than 80 µg. Openings are made after evaluations of current and historical records. Areas in which there is a high-value shellfish resource and where there is reason to believe that there will not be another rise in toxin may be reopened after at least two weekly samplings below 80 µg.

24.6.3 The need for harmonization

24.6.3.1 *General Agreement on Tariffs and Trade*

The World Trade Organization (WTO) emerged as a consequence of historical efforts and negotiations intended to achieve the liberalization of international trade. The WTO includes the General Agreement on Tariffs and Trade (GATT) and comprises about 125 countries representing more than 90% of international trade. The basic policy of the WTO is the progressive elimination of the barriers protecting domestic markets in the past. Within this framework, sanitary standards or regulations may be used as non-tariff barriers. The GATT agreements on sanitary and phytosanitary measures acknowledge the right of WTO members to establish regulations for health protection as long as these are based on scientific evidence and risk assessment and do not discriminate among members where identical or similar conditions prevail. "Sanitary regulations are not to be applied in a manner that constitutes a disguised restriction to international trade. Thus, when establishing a suitable level of sanitary protection, each country will consider the aim of reducing as much as possible the negative effects on trade so that regulations do not involve a restriction higher than that required" (Council Decision 94/800/EC). Based on GATT agreements, trade relationships shall be carried out based on the principles of harmonization, equivalency and transparency. This means the establishment of common sanitary measures up to international standards, the acceptance of different sanitary measures whenever it has been demonstrated that the appropriate level of protection has been achieved and the elaboration of regulations reflecting absolute transparency. WTO members should provide information on their sanitary regulations and notify any change. GATT agreements encourage the uniformity of sanitary regulations in order to eliminate risks to consumers and reduce obstacles to trade. However, when considering existing regulations for marine toxins, discrepancies between methods and limits are evident, even between countries for which a free trade and unrestricted circulation of seafood are already in place. Current attempts at harmonization in the EU, the APEC area and the USA are described below.

24.6.3.2 *European approaches to marine toxin control and harmonization*

A large number of European coastlines are affected by toxic events caused by harmful algal blooms. Toxic episodes due to PSP have been registered since the 1950s and DSP and ASP toxins have been the cause of prolonged closures of shellfish production areas. On the other hand, a new group of toxins, azaspiracids

(AZAs), responsible for the toxic syndrome Azaspiracid Poisoning (AZP), might affect a number of EU countries. At present, there are no records of shellfish contamination due to the presence of brevetoxins but microcystins and nodularin in molluscs have been reported occasionally which might represent an additional risk for shellfish consumers.

Within Europe, the EU is an area of free trade with unrestricted circulation of merchandise and border controls only for imports from countries outside the EU. Over the past decade, a number of Directives dealing with food safety legislation have been elaborated and implemented to ensure consumer protection, high quality of products and fair competition.

In the case of molluscan shellfish, the process of harmonization is at an advanced stage. EU Directives 91/492/EEC and 91/493/EEC lay down the health regulations for the production and placing on the market of live bivalves and fishery products respectively. Directive 91/492 establishes controls for the shellfish production areas and the different activities of harvesting, transport, depuration, storage, processing and marketing, as well as microbiological and chemical parameters and marine toxin content. Directive 97/61/EC modifies and complements Directive 91/492/EEC concerning traceability of mollusc batches from harvesting to marketing and includes domoic acid control. According to Directive 91/492/EEC, a public health control system must be established by the responsible authority, in order to verify whether the requirements laid down in the Directive are complied with. For marine toxins, this control system must include:

1. Periodic monitoring of live bivalve molluscs and production areas, in order to check the possible presence of toxin-producing phytoplankton in the waters and biotoxins in shellfish.
2. Sampling plans that take into account possible variations in production and relaying areas in the presence of toxic phytoplankton. The sampling must be carried out periodically to detect changes in the composition of the toxic phytoplankton populations and their geographical distribution. Information leading to suspicion of accumulation of toxins in mollusc flesh must be followed up by intensive sampling, increasing the number of sampling points and the number of samples. Toxicity tests will be carried out using the molluscs of the affected area which are most susceptible to contamination. If the results of a sampling plan show that marketing live bivalve molluscs may constitute a hazard to human health, the responsible authority must close the area producing molluscs until the situation has been restored to normal. Marketing molluscs from that area may not be re-authorized until new sampling has provided satisfactory test results.
3. Laboratory tests of the end product to verify that the level of marine biotoxins does not exceed safety limits.
4. Inspection of establishments at regular intervals.
5. Checks on storage and conditions of transportation for consignments of live bivalve molluscs.

Traceability is another important aspect regulated by Directive 97/61/EC. This implies the implementation of a registration and labelling procedure, so that, if a health problem occurs after placing shellfish on the market, the batch can be traced back to the dispatching establishment and the harvesting area of origin.

Regarding methods and limits for the control of PSP, DSP and ASP, Directive 91/492/EEC states:

> The total PSP content in the edible part of the shellfish must be lower than 80 µg g 100 g^{-1} of tissue, measured by a biological testing method that can be associated with a chemical acknowledged method. In the case of discrepancies, the reference method shall be the biological method.
>
> The normal biological testing method must not give positive results to the presence of DSP in the edible part of molluscs. The total ASP content in the edible part of the shellfish must be lower than 20 µg domoic acid g^{-1} by an HPLC method.

The Directive is vague and neither specific biological methods nor criteria of acceptability have been established for PSP and DSP control. In the case of DSP, the action limit is dependent on the detection limit of the biological method used. Concerning monitoring programmes, the criteria for the design of the sampling plans in relation to the ideal number and location of sampling stations, sampling points, sampling frequency and criteria for closing and re-opening the areas have not been established. The need to eliminate inconsistencies among EU Member States and harmonize European trade led the European Commission to nominate a National Reference Laboratory on Marine Toxins (EU-NRL) in each Member State and a Community Reference Laboratory (EU-CRL, Vigo, Spain) to co-ordinate the activities of the network of EU-NRL and create a forum for discussion and agreements on analytical methods, performance criteria and action levels for toxin control.) The tasks of the EU-CRL are to: 1) inform NRLs on methods and intercomparative testing, 2) co-ordinate the application of methods by organizing intercomparative testing, 3) co-ordinate the investigation of methods and inform on advances in the area, 4) organize training courses, 5) collaborate with laboratories from third countries 6) assist the European Commission in case of discrepancies, and 7) assist the NRL on accreditation activities.

The tasks of the NRL are to 1) co-ordinate the activities of the national laboratories, 2) assist the competent authority to organize the system for monitoring marine biotoxins, and 3) organize comparative tests between the various national laboratories and disseminate the information supplied by the EU-CRL to the competent authority and national laboratories.

The EU-NRL network interacts through annual meetings and by means of working groups hosted by the European Commission. These working groups deal with different marine toxin issues and are integrated by experts from the EU-NRL network and by outside experts when additional capabilities are called for. The working groups might deal with items related to the activities of the EU-NRL or with specific issues at the request of the European Commission or the Standing Veterinary Committee. The conclusions and/or recommendations of some working groups have been used as a pre-normative study for the elaboration of new regulations. In recent years, the EU-NRL network and the European Commission have made a big effort to achieve equivalent approaches for the control of marine toxins in shellfish. For PSP sanitary control, experts of the EU National Reference Laboratories on Marine Toxins agreed that the biological method mentioned in Directive 91/492/EEC should be that described in the AOAC Official Methods of Analysis (1990) and that the tolerated level should be that specified in the Directive. As some recently developed technologies such as the receptor binding assays and some immunoassays (MIST

Alert) have shown that they may be suitable alternatives to the mouse assay, the EU-NRL group intends to evaluate and assess these methodologies with a view to apply them if it is demonstrated that they provide a level of protection equivalent to that of the mouse bioassay.

Regarding DSP control, a working group performed a risk assessment on the basis of the available data on OAs PTXs, YTXs and AZP with the goal of recommending allowance levels and suitable detection methods. The working group's recommendations and conclusions formed the scientific basis of Decision 2002/225/EC establishing limits and methods for the control of OA, DTXs, PTXs YTXs and AZAs (see section 24.6.1). Similarly, an intense study carried out by another working group on ASP in scallops formed the basis of Decision 2002/226/EC, also discussed in section 24.6.1. The issue of sampling plans has also been addressed by the EU-NRL network and the conclusions of a working group may be included in the near future in EU regulations. Other current activities of the EU-NRL network include the preparation and distribution of toxins standards and reference materials, proficiency tests, inter-laboratory studies and validation of analytical methods.

In addition to the EU-NRL activity, it is worth mentioning the work of the European Standardization Committee (CEN), a committee made up of members of national standardization bodies from 19 European countries which produces standard methods both to enable food manufacturers to determine with reasonable certainty whether a production batch may be put on the market and to enable regulatory authorities to determine whether foodstuffs on the market comply with legal limits. Within CEN, the Working Group 5 – covered within Technical Committee 275 (Food analysis-Horizontal Methods) – selects and elaborates methods that are to become EU standards. CEN standards are not necessarily official EU methods but the European Commission may make use of them for substances subject to legal limits. The CEN Working Group 5 works closely with the EU-NRL network; both are currently elaborating a report on performance criteria for analytical methods for phycotoxins. The criteria will be based on the results of validation studies; published data and will include scope and definitions, tables with precision (RSD_r and RSD_R), recovery experiments for various phycotoxins and information about extraction solvents, applicability and food types.

EU procedure for international seafood trade

In relation to countries outside the EU, Directive 91/492/EEC states that provisions applied to imports of live bivalve molluscs from third countries shall be at least equivalent to those governing the production and placing on the market of Community products. Inspections shall be carried out on the spot by experts from the European Commision and the Member States to verify whether the conditions of production and marketing can be considered as being equivalent to those applied in the Community. In deciding on equivalency, particular account shall be taken of legislation of the country, organization of the responsible authority and inspection services, facilities for monitoring the implementation of their legislation, health conditions of production and marketing, monitoring of production areas in relation to microbiological and environmental contamination and the presence of marine biotoxins, information on toxic phytoplankton, in particular species not occurring in Community waters, and assurances on the compliance with the standards in Directive 91/492/EEC.

EU Decision 97/20/EC of 17 December 1996 establishes a list of countries outside the EU that fulfil the requirements for equivalence concerning production of bivalve molluscs, echinoderms, tunicates and marine gastropods. This decision has undergone modifications in recent years (Decisions 97/565/EC, 98/571/EC, 2000/332/EC, 2001/38/EC, 2001/255/EC). Imports from the countries listed in the Decision are allowed and, after veterinary control at the border inspection posts, products may enter the Community.

The list of authorized countries could be modified if new agreements of equivalence are signed or if the current agreements are modified. Information about EU legislation currently in force or in preparation is available at the website:

http://europa.eu.int

24.6.3.3 *APEC approaches to toxin control and harmonization*

A similar programme leading towards harmonization of sanitary controls is being carried out among the countries belonging to the Asia Pacific Economic Cooperation. APEC comprises 18 coastal and archipelago economies with highly diversified marine resources that constitute a significant portion of the regional economic production. Economies belonging to the network are Australia, Brunei, Canada, China, Chinese Taipei, Hong Kong China, Indonesia, Japan, Malaysia, Mexico, New Zealand, Papua New Guinea, Philippines, Republic of Korea, Singapore, Thailand and the USA. One of the APEC goals is to eliminate trade barriers and establish a free trade area by the year 2020 with unrestricted movement of merchandise, including fish and shellfish products. The occurrence of toxic phytoplankton is a common phenomenon in all APEC economies which has affected shellfish resources through impacts on public health and ecosystems. Economic losses have been severe in some countries of this region. Currently, there are great differences in the capabilities of the different APEC economies to cope with toxic algal events, and monitoring programmes differ in geographical coverage, resources monitored, procedures used, toxins analysed and regulatory levels. Some APEC countries have already developed extensive expertise, and are extremely well equipped with analytical instrumentation to cope with phycotoxins, as in the case of Canada, Japan, New Zealand or the USA, while others are still beginning to implement monitoring programmes and have not established regulations to restrict the harvesting or marketing of shellfish contaminated with phycotoxins. As these differences would constitute a serious obstacle for the establisment of a free trade area, a Red Tide/Toxic Algae Project has been set up by the Working Group on Marine Resource Conservation (MRC) in order to achieve common seafood safety standards and legislation. The project includes a large-scale regional programme whose main goal is to provide the training, infrastructure and supervision necessary to establish uniformity in policies, procedures and capabilities so that each economy can certify that its seafood is free from phycotoxins using procedures and standards that are acceptable to all economies within the region. Hence, fishery products can be exported and imported freely among the economies. Human resource development and technical exchange is met through focused working groups and task teams, technical workshops, symposia, exchange visits and consultant reports. Within the project framework, those countries with greater expertise and regulatory infrastructure will provide training and technical assistance to other APEC countries lacking the capabilities needed to face toxic events.

The fundamental principle for APEC economies in seafood certification and trade with respect to marine algal toxins is the application of performance-based

criteria wherever possible. The current prescriptive analytical methods are considered barriers to trade. This is a fundamental concern of APEC members. There is an urgent need to encourage member economies to develop and accept cost-effective, validated methods for analysis of seafood toxins that can be used for regulation in monitoring programmes without jeopardizing the trade in safe seafood. The equivalency mechanism can be effectively used to measure the performance of different member economy programmes in meeting agreed levels for seafood safety. This approach implies that technical regulations should be designed in such a way as to be performance-based rather than prescriptive in nature. Currently, there is a process for designating APEC Reference Laboratories for Algal Toxin Analysis and Standards to advise on analytical methods and standards, promote the development of standard reference materials and assist in the development of a distribution network for standards and reference materials.

A task team on Algal Biotoxin Regulation has proposed that all APEC economies adopt the following action limits:

- *For PSP toxins:* the internationally accepted limit of not more than 80 µg STX equivalent 100 g^{-1} consumable product.
- *For ASP toxins:* the internationally accepted limit of 20 ppm shellfish meat.
- *For NSP toxins:* the action limits currently used in the USA, which includes detectable NSP toxin levels in shellfish tissues, as measured by the APHA (1985) method, and *Karenia brevis* concentrations exceeding 5,000 cells L^{-1}. This is also the cell density that triggers public health warnings in New Zealand, which uses an action limit in shellfish tissues of 20 MU 100 g^{-1}. In the USA, harvesting areas are reopened when mouse bioassay results indicate that shellfish from closed areas contain <20 MU 100 g^{-1}.
- *For DSP toxins:* currently accepts the absence of toxins using bioassays (either rat or mouse) as the limit, subject to change as improvements occur in understanding of the toxins or in the availability of detection methods. Furthermore, the use of alternative detection methods is welcomed, insofar as the economy demonstrates that the chosen alternative does not perform less well than the bioassay.
- *Toxic plankton:* it is recommended that economies consider plankton monitoring as a tool that can help make toxicity monitoring more cost effective.

APEC and the EU are currently interacting to establish equivalent aproaches to phycotoxin control and regulations that eliminate obstacles to trade (APEC, 2000).

24.6.3.4 *US approach to harmonization*

Interstate Shellfish Sanitation Conference and National Shellfish Sanitation Program (ISSC/NSSP, USA)

In the USA, the Food and Drug Administration (FDA) is responsible for ensuring that all food items shipped in interstate commerce are safely prepared, packed and stored under sanitary conditions. Items must be correctly labelled. In 1925, the FDA was authorized to receive aid from state and local authorities to enforce laws to prevent and suppress the transmission of communicable diseases. This led to the creation of the National Shellfish Sanitation Program (NSSP). The NSSP includes members of the FDA, state control agencies and the shellfish industry. They set standards to ensure that sanitary conditions are respected in the production and interstate

shipment of shellfish, on a voluntary basis. The Interstate Shellfish Sanitation Conference (ISSC) formed in 1982 consists of members of state and federal control agencies, the shellfish industry and the academic community. A voluntary organization, it is open to all persons interested in ensuring that shellfish reach the consumer under safe, sanitary conditions. The ISSC provides up-to-date sanitation guidelines for the regulation of harvesting, processing and shipping of shellfish. It also provides a forum for all interested persons to air their concerns regarding shellfish sanitation and disseminates information on recent developments via publications, meetings and work with academic institutions as well as trade associations. Any country shipping shellfish to the USA must comply with these regulations (Hurst, personal communication).

Closure of contaminated areas

An area shall be closed to the harvest of shellfish immediately if the meats of shellfish harvested from that area contain 80 µg of Paralytic Shellfish Poison toxins per 100 g of shellfish meats or contain concentrations of other toxins or contaminates known to be harmful to consumer health. The Commissioner may also close surrounding areas of lower toxicity levels to provide a margin of safety in the event of rapidly changing toxicity levels.

The Commissioner may close areas or fisheries if sufficient current information is not available to assure above conditions do not exist or current information does not permit prediction that the above conditions are unlikely to occur (NSSP, 1990, Chapter 23.30).

Repeal of polluted or contaminated area closures

The Commissioner shall repeal polluted or contaminated area closure regulations when sanitary surveys reveal that pollution or contamination conditions no longer exist and that shellfish may be harvested from the area without threat to the public health (NSSP, 1990, Chapter 23.40).

Toxins other than PSP covered under NSSP Manual requirements, January 1990

The US Department of Marine Resources (DMR) acknowledges that there may be toxins other than PSP in shellfish. The DMR conducts a limited sampling programme for amnesic shellfish poison (ASP), domoic acid, in conjunction with its PSP sampling programme. Information from Canada concerning domoic acid is available on an up-to-date basis. Closures will be made whenever domoic acid levels reach 20 ppm. Diarrhetic shellfish poisoning (DSP) has not been reported from North America. Owing to the presence of Dinophysis spp., the DMR recognizes that cases are likely to occur but it cannot assay for DSP toxins at this time. Any area suspected of containing ASP, DSP or any other toxin defined or undefined will be closed under Chapter 23.30 until such time that the area is deemed toxin-free.

In order to sell shellfish to EU countries, the products must be accompanied by a Health Certificate. The FDA has indicated that these certificates will be issued if the dealer involved is in compliance with applicable laws and regulations. This will involve giving consent for the FDA to access quality control, production and other relevant records. If the dealer acts in compliance with NSSP regulations, the EU requirements other than those for DSP will be met. DSP may or may not be a problem if the dealer has any records concerning the presence of toxic algae in the harvest waters.

There is at present no test for DSP in the USA. It is recommended that anyone contemplating shipping shellfish to EU countries collect data from their harvest areas on the presence or absence of potentially toxic plankton. It is reasonable to expect the FDA to make the states assume responsibility for determining any realistic safety standards. If and when a dealer asks for certification for the EU market, it must be pointed out that there is no way of determining a negative DSP assay.

US procedure for international seafood trade

Those interested in selling shellfish on the international market should contact their National Health agency and National Natural Resource agency for current regulations governing shellfish. In order to market safe shellfish, regulations must be in place and enforced concerning the sanitary suitability of the harvest area(s) and the safe processing of the shellfish. The exporting nation is responsible for determining the safety of the shellfish being exported. If the potential shellfish market is the USA, there must be in place a Memorandum of Understanding (MOU) between the national agency responsible for shellfish safety and the FDA. Nations having current MOUs with the FDA are Australia, Canada, Chile, Iceland, Japan, Mexico, New Zealand, Republic of Korea and the United Kingdom. The MOUs may restrict harvest areas and selected species of shellfish. Shipments of shellfish to EU Member States must meet the EU sanitation requirements under Council Directive 91/492/EEC), which requires a Certificate of Health delivered by a competent authority from the nation of origin. This certificate must address measures ensuring that the shellfish meet EU standards for shellfish sanitation. Other countries may also require a MOU from the nation of origin concerning safety of shellfish being imported.

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25

Finfish mariculture and harmful algal blooms

J. E. Rensel and J. N. C. Whyte

25.1 Background

Over the past 25 years, biotoxin production and the physical damage caused by phytoplankton have become an increasing concern for fisheries resources. Worldwide there is a greater understanding of the diversity of harmful algal blooms and a growing awareness of the undesirable impacts of HABs on wild and cultured finfish (Hallegraeff, 1993; Moncheva *et al*., 1995; Elbraechter, 1996; Horner *et al*., 1997; Kim, 1997; Smayda, 1997; Whyte *et al*., 1997; Zhu *et al*., 1997; De Silva, 1999). The recent expansion of mariculture in both the northern and southern hemispheres has revealed several species of microalgae as potent fish killers.

This chapter focuses on causes of fish mortality from HABs, management measures for finfish mariculture (i.e. monitoring, prediction, remote sensing and planning) and the developing field of mitigation (i.e. reduction or removal of HABs at farm sites or measures to reduce impacts), although the categories are not mutually exclusive. Not dealt with are broad-scale or extensive control measures considered for removing HABs. Fish species of salmonids, yellow-tail tuna and red seabream affected by HABs are highlighted because of their economic importance in mariculture. Fish-killing microalgae that solely affect wild fish stocks are not discussed here, although over time these categories will tend to merge as mariculture expands into new locations and new marine fish species are farmed. Bruslé (1995) reviewed many of these HABs, but a sampling of more recent citations for wild and cultured fish kills includes *Alexandrium tamarense* (Montoya *et al.*, 1997); *Gymnodinium breve* (= *Karenia brevis*) (Warlen *et al*., 1998), *Gymnodinium pulchellum* (Steidinger *et al*., 1998*a*), *Pfiesteria piscicida* (Burkholder and Glasgow, 1997*a*, 1997*b*; Burkholder *et al*., 1999); *Karenia mikimotoi* (as *Gyrodinium aureolum*) (Blasco *et al*., 1996), *Phaeocystis pouchetii* (Aanesen *et al*., 1998; Stabell *et al*., 1999), and *Tetraselmis* sp. (Jones and Rhodes, 1994).

25.2 Economic impacts

Economic losses from HAB impacts on mariculture can be devastating to local or regional economies, but truly large-scale fish-killing blooms do not occur frequently in the major mariculture areas of the world. Major salmon-growing areas such as Norway, Chile and British Columbia, as well as other contributors such as Washington State, Tasmania and New Zealand, may record fish-killing events occasionally. These are typically regional events that seldom extend over larger scales. Mariculture fish may be affected by blooms for a variety of reasons, such as remote

geographical location, lower density of bloom organisms, smaller extent of bloom, success of management or mitigation efforts, etc. Mariculture is an important industry in Norway where fish farmers first experienced repeated fish kills in 1982 due to the unarmoured dinoflagellate *Karenia mikimotoi* (as *Gyrodinium aureolum*) (Dahl and Tangen, 1993). Wild fish kills from the same species in European waters had been reported before the introduction of net-pen culture in the 1960s (Andersen, 1996). Subsequently, several other HAB species (including *Chrysochromulina* and *Prymnesium*) caused massive fish kills in Norway (Dahl and Tangen, 1999).

Few detailed studies on the economic losses resulting from HABs have been conducted (Tomerlin and Adams, 1999), but a conservative estimate in the USA alone from 1987 to 1992 would be US$50 million per year (Anderson *et al.*, 2000). Fish farmers are reluctant, in general, to divulge losses suffered from HABs, which adds to the difficulty of estimating accurate economic impacts. Nevertheless, it is clear that the worldwide economic impact of HABs will escalate with continuing mariculture growth. World production of mariculture fish increased rapidly from 196,638 tonnes in 1984 to 694,697 tonnes in 1998, an annual linear increase of 31,747 tonnes per year (FAO, 2000). Over the same period the value of mariculture fish increased from US$835 million to US$3,027 million. Mariculture is a neophyte industry compared with freshwater aquaculture, so these figures represent only 3.5% and 10.4% of the total production and value, respectively, of the combined fresh water, brackish water and marine fish cultured worldwide (FAO, 2000). In recent years an extensive prolonged bloom of *Cochlodinium polykrikoides* in the summer of 1995 caused losses of US$95.5 million to the mariculture in the Republic of Korea (Kim, 1997). Similarly, this harmful alga caused losses of US$72,500 worth of mariculture grouper and black seabream in China (Qi *et al*., 1993), and for the first time in Canada caused about US$2 million in lost farmed salmon in 1999 (Whyte *et al.*, 2001). The same species was present in a mixed bloom in Hong Kong SAR and the South China Sea in 1998, when 80% of Hong Kong's 1,500 fish farms were severely affected by blooms thought to be linked to the new species *Karenia digitata* (Yang *et al*., 2001). The bloom caused great societal upheaval and the loss of 1,500 tonnes of mariculture fish worth an estimated US$32 million (Anon., 1998*a*, 1998*b*; Anderson *et al*., 1999; Yin *et al*., 1999). Economic losses of aquaculture fish from 1980–1990 in Japan amounted to about ×15.5 billion from Raphidophytes such as *Chattonella* spp. and *Heterosigma akashiwo* (Honjo, 1994). In the north-eastern Pacific, salmon farms in British Columbia and Washington State lost in excess of US$35 million during the late 1980s and 1990s to *H. akashiwo* and *Chaetoceros* spp. (Horner *et al.*, 1997; Whyte, 1997*b*, 1999*b*). Repeated HAB occurrences have largely extirpated commercial finfish aquaculture from some coastal regions, such as the Sunshine Coast on the Strait of Georgia, British Columbia. Salmon mortality was associated with the annual northerly advection of *H. akashiwo* following excystment from a presumed seed area near Jericho Beach, Vancouver (Taylor and Haigh, 1993). Clearly, with a continued increase in fish aquaculture worldwide, a greater understanding of harmful species will be required to alleviate the economic hardship they elicit.

Beyond the economic losses, fish and shellfish mariculture functions as a valuable indicator of the health of our coastal seas, like the proverbial 'canary in the mineshaft'. Fish reared in mariculture cages may be affected more than wild fish that have the freedom to move and evade encroaching harmful blooms. As discussed below, improperly located mariculture has the capability of actually stimulating

algal blooms, but there are effective strategies for dealing with nutrients and other wastes from net-pens that have been or are being adopted by governments (e.g. Kreeger, 2000; Rensel, 2001). HABs are a major technical and biological impediment to marine finfish aquaculture because of biotoxin accumulation in the flesh in some cases that may compromise public safety or because of substantial fish mortality. Catastrophic losses of farmed fish have occurred in recent years due to some HAB species and it is highly probable that new microalgal species will be implicated in future losses of aquaculture fish as the geographical range of mariculture expands.

25.3 Causes of fish mortality

Table 25.1 lists fish-killing microalgal species and the concentrations thought to be important for mariculture response action. It is emphasized that these action levels are gross approximations, and will vary with geographical location, fish species, fish size and condition, environmental conditions and clone differences (e.g. Marshall and Hallegraeff, 1999). Although much has been learned about the mechanisms responsible for fish death from HABs, there is still uncertainty and lack of definitive understanding concerning several important species and groups of fish-killing HABs. A variety of physiological mechanisms, singly or in combination, may lead to fish mortality. They can be generally categorized as

(a) physical damage or irritation of gill tissue leading to mucus production, blood hypoxia and possibly bacterial infection;
(b) toxigenic reactions to ichthyotoxic agents;
(c) blood hypoxia from environmental oxygen depletion;
(d) gas-bubble trauma from oxygen supersaturation.

The mechanisms are briefly summarized as follows.

Gill clogging, irritation or mechanical damage to the gill tissue triggers copious production of protective mucus (composed of glycoproteins), to relieve the abrasion of the gill epidermis or as a means of clearing the blocked filaments. Severe hyperplasia and necrosis of the epithelium may result, leading to blood hypoxia and respiratory dysfunction as the ultimate cause of death (Rensel, 1993; Yang and Albright, 1994*b*). Sublethal cell concentrations <5 cells ml^{-1} may lead to increased susceptibility to infectious disease, such as vibriosis, due to the suppression of the fish's immune response (Albright *et al.*, 1993). Diatoms known to cause mucus production are principally *Chaetoceros concavicornis* and *C. convolutus*, but other diatoms such as *Skeletonema* or *Thalassiosira* spp. and the silicoflagellate *Dictyocha speculum* are equally effective in stimulating excess mucus production in fish gills when present in dense concentrations.

Mechanisms of fish death due to ichthyotoxic agents are much less well-defined, and may vary considerably among different genera of HABs. In some cases, they can produce gill lesions and severe epithelial separation of the lamellae and filaments from reactive oxygen species (Yang *et al.*, 1995; Ishimatsu *et al.*, 1996*a*, 1996*b*; Kim *et al.*, 1999). Hepatotoxicity by microcystin-like compounds has been reported (Kent *et al.*, 1996), but the etiology of this condition is poorly understood. Neurotoxicity by brevetoxins produced by *Karenia brevis* is well known (Baden, 1989). Hemolytic or blood agglutination by bioactive substances produced by *Chrysochromulina polylepis*, *Alexandrium tamarense* and *Prymnesium* spp. have

TABLE 25.1 Harmful phytoplankton species known or suspected of causing fish losses in mariculture and recommended action concentrations. We emphasize that most cited cell concentrations are gross approximations and will vary considerably from case to case. This approach inspired by a report by Kim (1998*b*). Recent name changes (Daugbjerg *et al*., 2000) are included here in parentheses, synonyms are in brackets

HAB category and species	Action level to intensify management	Action level to initiate mitigation	References
Diatoms and mixtures			
Chaetoceros concavicornis, *C. convolutus* and other subgenus Phaeoceros, possibly *C. danicus*	>2–5 cells ml^{-1}	>5 cells ml^{-1}	Bell (1961); Bruno *et al.* (1989); Rensel (1992, 1993); Taylor (1993); Albright *et al*. (1993); Taylor and Horner (1994); Yang and Albright (1994*b*)
Leptocylindrus minimus	1,000–10,000 cells ml^{-1}	>10,000 cells ml^{-1}	Clément (1994); Clément and Lembeye (1993)
Other harmful diatoms including *Chaetoceros* subgenus Hyalochaete, and in some cases *Corethron criophilum*, *Skeletonema costatum*, *Thalassiosira* spp.	>50,000–100,000 cells ml^{-1}, depending on sensitivity of fish and life stage	>100,000 cells ml^{-1}, especially if juvenile fish reared	Kim (1998*b*); Speare *et al*. (1989); Kent *et al*. (1995)
Other diatom and dinoflagellate mixtures	>40,000 to 80,000 cells ml^{-1} if >50% dinoflagellates	>80,000 cells ml^{-1} if >50% dinoflagellates	Kim (1998*b*)
Dinoflagellates			
Alexandrium tamarense	Unknown; acute mortality to farmed fish not well documented and may not occur; mortality and potential food web toxin accumulation for wild fish		Mortenson (1985); Erickson (1988) (for *A. catenella*)
Ceratium fusus	Unknown; gill irritation, poorly understood, affects oyster larvae and shrimp		Rensel and Prentice (1980); Cardwell *et al.* (1979)
Cochlodinium spp. *C. polykrikoides*	300 to 1,000 cells ml^{-1}, aeration induces lethality	1,000 cells ml^{-1}	Kim (1998*a*); Yuki and Yoshimatsu (1989); Whyte *et al.* (2001)
Gymnodinium breve (*Karenia brevis*) [*Ptychodiscus brevis*]	5–10 cells ml^{-1}, sometimes co-occurs with *G. mikimotoi*	>10–25 cells ml^{-1}	Ray and Wilson (1957); Steidinger *et al*. (1998*b*)

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TABLE 25.1 *(Suite)*

HAB category and species	Action level to intensify management	Action level to initiate mitigation	References
Dinoflagellates			
Karenia digitata	Unknown, order of magnitude estimates possible		Anderson *et al.* (1999)
Gymnodinium mikimotoi (*Karenia mikimotoi*)	1,000–3,000 cells ml^{-1}	>3,000 cells ml^{-1}	Kim (1998*b*)
Gyrodinium aureolum (*Karenia mikimotoi*)	500–2,000 cells ml^{-1}	>2,000 cells ml^{-1}	Kim (1998*b*); Takayama and Adachi (1984); Okaichi (1989); Tangen (1977)
Noctiluca scintillans	Un-ionized ammonia depending on pH and temperature		Okaichi and Nishio (1976)
Prymnesiophyte flagellates			
Chrysochromulina polylepis, C. leadbeateri	Unknown; causes gill damage and osmoregulatory problems, threshold of damage not defined		Estep and MacIntyre (1989); Aune *et al*. (1992); Skreslet *et al*. (1993)
Phaeocystis pouchetii	Unknown; irritant substances and the alga's mucus can clog gills		Lancelot *et al.* (1987); Gaines and Taylor (1986)
Prymnesium parvum, P. patelliferum	Unknown; toxins cause tissue, blood-cell and neurological damage		Shilo (1982); Guo *et al*. (1996); Larsen and Bryant (1998)
Raphidophyte flagellates			
Chattonella antiqua (possibly *C. marina*)	1–50 cells ml^{-1}	Conflicting data: 50 to 500,000 cells ml^{-1}, may be dependent on fish species, size, etc.	Okaichi *et al.* (1989); Onoue *et al.* (1990); Tanaka *et al*. (1994); Kim (1998*b*); Hallegraeff *et al*. (1998)
Heterosigma akashiwo [*Heterosigma carterae*]	>50 cells ml^{-1}, less if very calm and warm weather	Variable to non-toxic, some cases, in others >750–1,000 cells ml^{-1}	Black *et al*. (1991); Taylor and Haigh (1993); Chang *et al.* (1990, 1993)
Silicoflagellates			
Dictyocha speculum [*Distephanus speculum*]	Unknown; siliceous skeleton or naked stage may irritate gills causing mucus production, oedema and hyperplasia and probable reduced oxygen and carbon dioxide exchange		Thomsen and Moestrup (1985); Larsen and Moestrup (1989); Erard-Le-Denn and Ryckaert (1990)

Source: Adapted from Anderson *et al*. (2001).

been documented (e.g. Edvardsen *et al*., 1990; Meldahl and Fonnum, 1993; Simonsen *et al.*, 1995; Simonsen and Moestrup, 1997; Igarashi *et al*., 1998). The actions of these algal bioactive metabolites on fish result in physiological damage to gills, major organs, intestine, circulatory or respiratory systems or interference with osmoregulatory processes.

The cause of fish death from exposure to raphidophyte blooms such as *Heterosigma* and *Chattonella* remains controversial and inconclusive. Histopathology of gills of moribund sub-adult salmon from net-pen kills usually show major damage to the epithelium and mucus build-up, but in one case with juvenile fish a labile ichthyotoxin was suspected as the cause of mortality (Black *et al*., 1991). Hallegraeff *et al*. (1998) noted that early evidence suggested that these algae produced a fatty acid that destroyed gill tissue. Subsequently, the focus shifted to production of reactive oxygen species (e.g. Oda *et al*., 1997, and many others), which may explain damaged gills seen in some cases. Superoxide anion radical and hydrogen peroxide produced by the alga reportedly strip the fish gill of mucus and lead to fatal osmoregulatory stress (Tanaka *et al*., 1994). This does not, however, fit with current knowledge of salmonid physiology because the normal, unstressed condition is to have little or no mucus on the gills (Handy and Eddy, 1991). Twiner and Trick (2000) further elucidated the possible physiological mechanisms for production of hydrogen peroxide. Twiner *et al*. (2001) concluded that production rates of hydrogen peroxide are orders of magnitude less than that required to cause mortality of selected vertebrate cell lines or *Artemia salina*, casting doubt on its role in fish deaths. Brevetoxin-like compounds in raphidophytes have been reported to produce respiratory and/or cardiac paralysis (Endo *et al*., 1992; Khan *et al*., 1996, 1997).

The etiology of fish death due to members of the *Gymnodinium–Karenia* complex of dinoflagellates is not well described. The available evidence, however, suggests that toxins are not taken up and retained in the tissues of fish killed by these species of toxic algae. Some low levels have been detected in fish killed by *G. breve* in Florida. A problem with these species involves their delicate structure, so the cells rupture easily when pumped through the fish gill chamber or when mariculturists attempt to filter or pump the water with the alga present. Fish mortality from many toxic HABs occurs quickly in most cases although toxins are *apparently* not transferred to the body muscle but could be a risk to consumers of the affected viscera. Further study is necessary in this area, as there may be much controversy and confusion about seafood safety during and after major fish-killing HAB events.

Environmental oxygen depletion through bloom senescence can lead to death from blood hypoxia (Jones and Rhodes, 1994). In Hong Kong waters, for example, these types of bloom far surpass toxic blooms in terms of occurrence and costs to fisheries or mariculture, with the exception of the massive fish kills from the spring 1998 bloom (Anderson *et al*., 1999). Oxygen requirement and physiological response to hypoxia is specific to fish species or family (Hishida *et al.*, 1998). Gas-bubble trauma and gill lesions produced by oxygen supersaturation from algal photosynthesis can occur (Renfro, 1963; Hishida *et al.*, 1999). Although these causative mechanisms will lead to fish death, the actual lethal doses, the synergistic lethality of sequential or mixed blooms of different species, and the precise mode of action of uncharacterized ichthyotoxic agents, remain uncertain and require considerable detailed study to allow more efficient management and mitigation in the future.

25.4 Management of HAB issues in finfish mariculture

25.4.1 Introduction

The occurrence of HABs is sometimes unpredictable, but the effects on fish farms may be successfully avoided or managed by a variety of means. In practice, fish farmers in areas not subject to recurring HAB events are often ill-prepared to deal with fish kills. Subsequent to major fish losses, government or industry should prepare plans to manage HABs, and consider local conditions, resources and abilities. Conceptual or mathematical models can be developed through hindcasting of empirical data and other means that may give indications of periods of increased HAB risk (Franks, 1997; Anderson *et al.*, 2001). Remote sensing from buoys, aircraft, satellites, and biosensors that provide sea surface temperature, current data, salinity, chlorophyll *a*, and characterization of the species can help track bloom advection into or through coastal areas with aquaculture facilities, as discussed below. Often more than one of the methods listed below will be employed, depending on the type of HAB, the fish species and the site location characteristics. Selection of method must be tailored to local hydrographic conditions (e.g. depth, current velocity, mixing properties) and the nature of the harmful algae (e.g. vertical distribution, mode of fish-kill action) likely to occur. Fish losses may occur very rapidly, so advance preparation of a management plan is recommended and systems must be ready to deploy at a moment's notice.

25.4.2 Environmental factors related to site selection

Site selection is one of the most important criteria for successful finfish mariculture, both in terms of the success of a project and for environmental protection. In the pragmatic world of fish farming, there are many important siting and permit considerations that may take precedence over HAB concerns. Environmental impact of large-scale mariculture, especially benthic impact, is now routinely monitored and regulated in many countries and has been the subject of extensive research in the past 30 years. Some areas have definitive and comprehensive performance criteria for fish farms for both water column and benthic impact that are the result of years of annual monitoring work (e.g. Rensel, 2001). Fish farms have been located by trial and error with respect to HABs, but there have been exceptions where prior studies have been effective for avoiding unsuitable locations. General aquaculture siting references include Edwards (1978), Sedgwick (1982), Beveridge (1987) and ICES (1992), but HAB management planning is best done on a local and regional basis.

Basic hydrographic monitoring should be conducted in the general vicinity of proposed fish mariculture in order to evaluate site suitability for the fish species to be reared, minimize benthic and water-column impacts, and estimate HAB risks. Vertical profiles of water temperature, salinity, dissolved oxygen, water transparency and chlorophyll *a* during high-risk periods can be surrogate indicators of the potential for certain types of harmful bloom. Concurrently collected phytoplankton samples for composition and density estimates from discrete depths are desirable to characterize a potential site, as some HABs tend to recur in the same areas. Sediment collection to identify dinoflagellate or microflagellate cysts may be useful in some cases to locate possible seed areas for harmful species, but this is typically not practised.

In temperate waters, large-scale mariculture should be located in moderate to deep-water areas where currents disperse solid and dissolved wastes adequately (Aquametrix Research Ltd, 1993). Shallow embayments, fjords that have nutrient-stressed surface waters or semi-blocking sills that significantly restrict flushing, are not recommended. Highest consideration should be given to areas already replete with dissolved inorganic macronutrients where other factors such as light and vertical mixing limit primary productivity. Proximity to neighbouring areas with soft bottom substrates that may harbour cysts of harmful algae is also a negative consideration. Hydrographic and phytoplankton data from prior studies by universities, government agencies and/or first-hand experience from local residents may be available at little or no expense to evaluate HAB risks in a candidate area. If these are not available, field surveys should be conducted during the appropriate algal bloom seasons. Alternatively, only small-scale or test facilities such as small net-pens should initially be operated while hydrographic, phytoplankton, fish growth and survival data are gathered.

In general, adverse effects of HABs may be reduced or avoided by selecting marine aquaculture sites with moderate or greater vertical mixing and tidal current velocity. Dinoflagellate and microflagellate blooms tend to dissipate in turbulent areas and cell growth is reduced (White, 1976; Whyte, 1997*a*). Additionally, strong currents will reduce or eliminate bottom sedimentation and adverse water-column effects from aquaculture waste products. Strong vertical mixing may have negative aspects, however, as many harmful flagellates that normally dwell near the surface can be distributed over a greater range of depths and prevent effective use of mitigation measures (discussed below). Moreover, these algae may accumulate near the surface during neap tidal periods and calm weather even in normally well-mixed areas. Similarly, diatoms such as *C. concavicornis* and *C. convolutus*, which are harmful to fish at very low concentrations, may be present throughout a well-mixed or actively mixed water column (Rensel Associates and PTI Environmental Services, 1991). The same species may occasionally be layered in dense concentrations near the surface in nearby quiet bays (Horner *et al.*, 1997). Despite extensive site surveys and the location of facilities in strong current areas, there remains the risk of blooms occurring during neap tides and calm conditions or bloom advection into a usually safe area from offshore or adjacent waters. Examples include *Karenia mikimotoi* blooms along the coast of Norway (Dahl and Tangen, 1990, 1993), shellfish toxin-producing *Gymnodinium catenatum* blooms on the northern Atlantic coast of Spain (Fraga *et al.*, 1988) and *Heterosigma akashiwo* blooms in active tidal channels of the central and northern coast of British Columbia (Haigh and Taylor, 1990; Taylor, 1993).

Nutrient enrichment from fish-farm waste has been associated with increased phytoplankton growth and the occurrence of algal blooms, mainly for farms located in shallow, poorly flushed sites that are sensitive to nutrient additions. Increasingly, this type of mariculture location is rarely sanctioned for new sites in both developing and developed economies (Folke *et al.*, 1994), although it is common in some Asian and other economies for existing sites. Models that predict the contribution and dispersion of organic and inorganic waste from fish farms can be used as management tools for siting farms and evaluating the potential for enrichment of the benthic ecosystem (Hevia *et al*., 1996; Silvert and Sowles, 1996). Gowen and Ezzi (1994) documented fish-farm-related nutrient enrichment in a Scottish sea-loch basin and found no change in species composition or increase in phytoplankton biomass. Water quality and phytoplankton monitoring of fish-farm sites in Ireland and Chile

found no evidence that salmon aquaculture caused phytoplankton blooms (Hensey, 1992; Bushmann *et al.*, 1996). A study in British Columbia demonstrated that salmon farms did not increase the food for mussels grown adjacent to the farms and that neither a direct contribution of nutrient in the form of fish feed or faeces nor an indirect contribution of phytoplankton from nitrogen enrichment was evident (Taylor *et al.*, 1992). Similarly, in the Bay of Fundy, Martin *et al*. (1999) could find no connection between fish farming and the occurrence of HABs. Marine fish farms in Washington State are required to locate in nitrogen-replete waters (SAIC, 1986; Rensel, 1989; Rensel, 2001), where other factors such as light and associated vertical mixing limit phytoplankton production (Rensel Associates and PTI Environmental Services, 1991). In that area, it is apparent that fish farms generally do not cause algal blooms including those of *Heterosigma*; rather, seasonally varying and local environmental conditions are responsible. These include nutrients and vertical stratification from runoff of major rivers, and local wind and rainfall, which set up oceanographic conditions, such as water-column stability, that may promote or exacerbate algal growth (Rensel, 1989; Parametrix, 1990; Rensel, 1995; Winsby *et al.*, 1996 and references therein; Horner *et al*., 1997; Connell and Jacobs, 1999).

Fish farms may be blamed for algal blooms because they are rigorously monitored for phytoplankton and the harmful species are often observed at the farm sites before the cells or their effects are seen elsewhere. In several instances, fish-killing HAB species were already present and killing fish prior to the introduction of fish mariculture (e.g. *Heterosigma* in the north-eastern Pacific). In some cases of poor site-location, HABs indeed may have been further stimulated by the introduction of mariculture wastes. But in all these cases (e.g. Seto Inland Sea, Japan; Tolo Harbour, Hong Kong; coastal areas of the North Sea) terrestrial and riverine nutrient sources far outweigh mariculture sources. In all cases, nutrient loading or shifts in nutrient ratio availability, while important, do not appear to be the only factors resulting in repeated or severe HAB occurrences.

25.4.3 Monitoring practices at mariculture sites

Routine monitoring of HABs is practised at fish-culture facilities in several countries to aid in predicting the onset of blooms or for direction of management or mitigation. Monitoring varies from occasional, simple, qualitative microscopic examination of plankton net-tow contents to detailed, daily species counts from water bottle or composite depth samples and remote sensing of chlorophyll by automatic profiling probes and aerial surveys. A few manuals for aquaculturists are available, including Gaines and Taylor (1986), Larsen and Moestrup (1989), Hallegraeff (1991) and Horner (1998). General phytoplankton monitoring strategies are discussed by Venrick (1978), Margalef (1978) and Smayda (1995). Some information is available regarding the concentration of HAB cells that cause injury or death of various species of cultured fish (summarized in Table 25.1). However, experience indicates that the lethal concentration varies with many parameters that include species, size, physiological condition and stocking density of fish, and environmental conditions such as water temperature, salinity, exposure periods, strain of alga, growth phase of alga, etc. In some cases, the mere presence of a few HAB cells in a non-concentrated water sample is reason for concern and an increase in monitoring efforts (e.g. harmful *Chaetoceros* spp.). At other times no fish mortality has been observed when concentrations of some HAB species, such as *Heterosigma akashiwo*, are relatively

high, suggesting that environmental factors may trigger lethality or that genetic variation among algal strains, and other possible cofactors such as differing bacterial assemblages, may explain the lack of toxicity (Doucette, 1995; Carrasquero-Verde, 1999).

After initial training and practice, fish-farm technicians, using light microscopes, are highly capable of identifying and counting several microalgal species that may be harmful to finfish. In practice, it is advantageous to have a regional centre of excellence for microalgal identification that interfaces with the farm technicians. Identification may focus on morphology, colour, size, swimming behaviour or appearance after fixation, but the use of dichotomous keys by technicians is uncommon. Fish farmers will often devise innovative counting techniques, such as the use of wet mounts on microscope slides of 0.1 ml sample water that slows down flagellate movement sufficiently to allow a detailed count, but does not completely inhibit movement or alter morphology (e.g. *Heterosigma* counting by fish farmers in the Pacific Northwest). Permanent microscope slides or photographs of key species found in manuals are useful, and Palmer-Maloney or Sedgewick-Rafter counting chambers are often used (Throndsen, 1995), although other means, such as filtration of larger volumes of water on to filter paper for detecting harmful diatoms, may be utilized. Fish culturists may establish routine HAB-monitoring protocols including the daily collection and storage of preserved water samples. Because blooms may develop or be advected into the farm site quickly, daily archiving of samples may help in predicting bloom formation in the future, particularly if matching hydrographic data are collected. If not analysed, preserved samples should be kept for at least two weeks before sample disposal and reuse of the bottles. Intensified monitoring is typically practised during peak risk periods or at any time when harmful cells are detected at threshold levels of concern (Table 25.1).

Aerial surveys of surface-oriented algal blooms can be an effective early warning system for mariculturists, if conducted by trained or experienced observers. Fish farmers are currently doing this in the Puget Sound region, routinely during the *Heterosigma akashiwo* growing season and more intensively on an emergency basis (Anderson *et al.*, 2001). These surveys involve small, fixed-wing aircraft surveys in conjunction with regular monitoring at farm sites and occasional 'ground-truthing' of aerial observations by sample collection and analysis. Observers sketch the distributions of blooms on a chart, but digitizing of results and GIS systems offer another means of managing the data. Results vary depending on wind conditions and other factors, but are considered adequate for mariculture early warning purposes. This system may not be applicable to fish-killing HABs that may not be as dense or surface-oriented. If a region's aquaculture resources are adequately developed, it is useful to form co-operative monitoring programmes for sharing information among the aquaculturists, with the use of electronic communications for rapid notification if the local risks from HABs are significant (Eslinger *et al.*, 1999). Some countries (e.g. Norway) have elaborate and co-ordinated phytoplankton monitoring for fish farms that is commensurate with the risks and potential losses due to fish kills. Other areas have monitoring responsibility resting with individual farms or companies. The latter solution is reasonable in many cases because farms are often hydrographically remote, which reduces the chance of area-wide harmful algae effects. Chile is the only country with an industry-wide, co-ordinated monitoring programme fully paid for by a mariculturists' association; in most other countries individual growers or corporations conduct their own programmes. In many cases, government is

initially involved in the instigation or co-ordination of monitoring programmes, but in corporate mariculture the industry generally takes over the responsibility either within or among competing fish-farming companies.

For large-scale bloom monitoring, sea-surface temperature, chlorophyll and other parameters may be monitored by satellite (e.g. NOAA CoastWatch in the USA). Passive optical sensors can measure ocean colour or the penetration of irradiance in surface water. Such sensors on ocean buoys can measure radiometric quantities and biological variability from minutes to months. In addition, new types of fluorometer can characterize the abundance, pigmentation and physiological state of phytoplankton (Cullen *et al.*, 1997). Instruments analysing absorption spectra *in situ* on moorings for remote sensing of HABs are being developed (Roelke *et al.*, 1999). However, many hurdles remain in optical monitoring and forecasting systems for HABs (Schofield *et al.*, 1999). A piezoelectric immunosensor, comprising a gold-coated piezoelectric crystal coated with a monoclonal antibody with highly specific reactivity to cytoplasmic membranes of *Chattonella marina*, was able to detect 100–10,000 cells ml^{-1} of the alga directly in seawater (Nakanishi *et al.*, 1996). The specificity of immunosensing detectors and microprocessors holds considerable promise for HAB detection and the ability to monitor advection of blooms towards mariculture areas. Prototype buoys for automatic HAB detection are currently being tested and deployed (See Chapter 5.). It is very likely that affordable buoy systems will be available for detection of HABs and that these buoys will be used in the future as fish farmers turn to offshore areas for sites. For small-scale mariculture in less-developed economies, however, more basic systems will remain a priority for the near future unless there is a need to protect other marine resources in these areas. Such applications could include surface meteorological observations, oil-spill contingency and oil-spill forecasting, environmental monitoring and documentation. A good example of an integrated bloom-monitoring programme for mariculture is that which covers most of the coastal waters and fjords of Norway (Andersen, 1996; Anderson *et al.*, 2001). Information on the occurrence of HABs is gathered from different sources including the 'fish-farm observation network' covering 40 sites along the Norwegian coast, fish farmers, aquaculture research stations and lighthouses, as well as mussel farmers. Furthermore the Norwegian Food Control Authority, as well as offshore SEAWATCH buoys, collect and transmit required data (see Chapters 21 and 22). The buoys currently measure a range of physical and biological parameters (wind speed and direction, air pressure, air temperature, wave height and period, current speed and direction, light attenuation, oxygen saturation, water temperature, salinity and radioactivity). The different sensors are located at 3 m depth, except the 11 temperature sensors that are situated on a cable extending from the surface to 50 m. It is anticipated that rapid development of new and better optical sensors, if adopted by the SEAWATCH manufacturer, will make this system a truly useful and practical means for early warning for HAB monitoring in coastal waters.

It may be possible to detect the onset of a HAB by monitoring fish behaviour. In some cases fish will reduce or stop feeding, seem lethargic, fall back in water currents, or may orient themselves unusually in the water, often swimming close to the surface and losing their self-righting ability. Different-sized fish or species of fish may react with differing behaviour patterns and physiological responses, and some related fish species are known to be more susceptible to HABs than others. Atlantic salmon, *Salmo salar*, are generally more susceptible to the effects of

Heterosigma akashiwo than the Pacific salmon *Oncorhynchus kisutch* or *O. tshawytscha*, with lethal effects more prevalent in the larger size classes (Black, 1991). Warm-water fish that may be sensitive bioindicators for the rest of a mariculture fish population subjected to environmental hypoxia include the red seabream, *Chysophrys major*, and the gold-line seabream, *Rabdosarga sarba* (Wu, 1990).

25.5 Mitigation strategies at mariculture sites

25.5.1 Introduction

Mitigation is defined *sensu stricto* as means taken to reduce HAB blooms or their effects (Jenkinson, 2000). The subtleties of definition are lost on mariculturists, who simply want to keep their fish alive and are generally not interested in broad initiatives to control or eliminate HABs. Mitigation of HABs is a controversial subject, but usually not a mariculture priority until recurring blooms occur in a given area. The use of chemicals to control HABs was initiated and largely abandoned decades before the recent rise of the mariculture industry (e.g. Rounsefell and Evans, 1958). Now a number of physical and chemical measures are being proposed, but most have not been evaluated with regard to collateral effects on other marine species or water and sediment quality. Nevertheless, at least a few of these techniques will probably emerge as economically and environmentally viable approaches for use in near-field (e.g. in immediate fish-farm vicinity) or far-field (extensive, broad-scale) mitigation of HABs. Here, we focus on the former, beginning with relatively simple practices used by many fish farmers worldwide.

25.5.2 Airlift pumping of deep water into cages

This technique may be effective when a harmful bloom is restricted to the near-surface layer of the water column. Air injected into vertically positioned large pipes with manifolds at the surface can be used to pump relatively cell-free water from depth to the surface of the pens. Airlift efficacy can be monitored by semi-conservative or conservative tracers, such as water temperature or salinity, to evaluate the effectiveness of moving water from depth to the surface. In some cases airlift pumping has been very useful in mitigating the adverse effects of *H. akashiwo* when the pens are surrounded by perimeter tarps or skirts. These can reduce the advection of HABs into the cages and reduce the dissipation of pumped water. Airlift pumps are a very cost-effective means of moving water, but must be used with caution as gas supersaturation can result if the inflow of air is located too deep within the pipe. Gas-bubble trauma caused by excessive gas supersaturation may also be avoided by using large-size bubble diffusers that act more to move the water than to increase dissolved gas concentration (Huguenin and Colt, 1989). Disadvantages of wide-diameter pipes to upwell water are the need to use cranes to lift and lower the pipes into position during the operation of the farm, and their horizontal drag on the floating farm caused by tidal currents. Air diffusers are easier to handle and less expensive to operate.

Some salmon net-pens in western Canada are equipped with perimeter skirts (~14 m tarps) and aeration systems using air diffusers that have proved effective in destratifying the water within the enclosed net-pen by upwelling of colder water and displacing microflagellates such as *H. akashiwo* that accumulate near the surface in

many cases (Anon, 1997*b*; Whyte, 1997*a*, 1999*a*). The use of perimeter skirting is not universally appropriate for all species. *Cochlodinium* sp., the dominant killer of fish in British Columbia in 1999, exhibited a deeper vertical migration than *H. akashiwo*, with vertical migration to 25 m during the night. Upwelling of high concentrations of cells occurred when tarps were deployed and air diffusion was activated. This practice did not alleviate the extent of mortality and limited its use for this harmful species. A strategy of raising the tarps in the evening to flush any residual cells from the pens during the night, then lowering the tarps when cell density increased on the surface in late morning, proved more successful in mitigating the effects of *Cochlodinium* sp. (Whyte *et al.*, 2001).

25.5.3 Oxygenation and aeration

Several existing aeration technologies are available to prevent fish mortality from environmental hypoxia, including aeration from coarse bubble air stones, venturi/aspiration nozzles, paddlewheels, etc. Coarse bubble aeration from air stones deployed below open net pens serves to create vertical convection of the water and does little to flush microalgal cells from skirted pens (Kils, 1979). Extremely vigorous aeration could help to break the chains and setae of the harmful diatom *C. concavicornis* into smaller sections that have proved to be less harmful in the laboratory (Rensel, 1992). However, the amount of turbulence required would stress and adversely affect the fish. Oxygenation to supersaturation levels may also be feasible in limited cases, where gill clogging or mucus production along with gill damage occurs, with or without toxins. The method has not been used on a commercial scale but could be used in net-pens equipped with perimeter skirts to retain the oxygenated water. Moderate levels of oxygen supersaturation may be beneficial to salmonids and yellow-tail subjected to compromised water quality conditions (Boyd and Watten, 1989; Okaichi *et al*., 1989). 'Moderate' in this case means <300% of air saturation concentration of oxygen without supersaturated nitrogen gas for 190 g rainbow trout (Boyd and Watten, 1989). Colt *et al.* (1991) recommend high-density salmonid culture with up to 400 mm Hg oxygen pressure. Supersaturated oxygen in fish-culture water has been used with some success in freshwater fish hatcheries and is often referred to as 'oxygen supplementation'. It is also one of the key factors in design of on-shore marine salmon farms that must rear larger fish at very high densities to be economically feasible. Although oxygen supersaturation has proved effective in the laboratory in reducing fish losses due to at least one harmful alga (*C. concavicornis*), the economics of supplying an entire net-pen facility with large volumes of oxygen are not favourable (Rensel, 1992). It is recommended that other measures should first be considered to mitigate the effects of HABs on finfish reared in net pens, unless the fish are very valuable such as brood stock of endangered species. Oxygenation of fish-culture water by aeration is not recommended to sustain marine fish exposed to HABs, because it is often only marginally effective in increasing the ambient concentration of dissolved oxygen during the blooms. This is because the transfer rate of dissolved oxygen to the water is proportional to the difference between ambient and desired concentrations. The exception would be during the senescent phase of very high-density blooms that in some cases may result in environmental hypoxia at night due to algal respiration and cell decay.

25.5.4 Moving net-pens

Towing net pens from an area affected by HABs to a known refuge area is one of the most effective mitigation measures and is a preferred method in some regions. It does, however, present considerable risk and expense, particularly for larger systems. The cost of towing net pens may be substantial, although a fish farm's insurance company may offset part of the expense. Towing involves the risks of structural damage to facilities, the escape of fish, and the death of fish by crowding and stress when the lower part of the net pens collapse in the currents if not towed properly. Interference with commercial shipping is another potential issue. A towing contingency plan should be devised in advance that includes protocols for dealing with anchoring systems, timing of movement with regard to tides, and approval by government agencies if necessary. Practice towing exercises may be warranted too.

Towing of net pens has been used for preventing salmon losses due to *H. akashiwo* in Puget Sound, Washington State and British Columbia (Horner *et al.*, 1997; Whyte, 1997*a*) as well as with the harmful dinoflagellate *Karenia mikimotoi* in Norway and *Chattonella antiqua* in the Seto Inland Sea of Japan and Hong Kong (Anderson *et al*., 2001). Aerial surveys by small aircraft or visual surveys by boat can be useful for detecting possible refuge areas, particularly when conducted in the morning before land breezes have dissipated HAB cells from surface water. Harmful *Chaetoceros* events in vertically mixed water bodies are difficult for fish farms to manage by towing because the vertical and horizontal distribution of the cells in the water column is not easily ascertained and cell concentrations that cause fish death may be relatively low. Finding a true refuge area may therefore be difficult.

25.5.5 Submerging net-pens

In the early days of mariculture, submersion of net-pens to avoid HABs was generally not recommended because it was technically difficult and net-pen cages were usually not equipped to deal with the different structural stresses of submerged nets. Additionally, physostomous fish such as salmonids must occasionally imbibe air for their float bladders to stay neutrally buoyant, which would not be possible during continuous captive submersion. However, in cases where cages are located in physically quiescent areas, or with specially designed pens with adequate site depth, the technique may have considerable merit. As fish mariculture evolves into growing a large variety of other marine fish, the opportunities for submersible or totally submerged pens are now becoming technically and economically feasible. Newer, large-scale pens specifically designed for open-ocean environments, which can be operated at the surface or submerged rapidly (e.g. Ocean Spar Technologies 'Sea Station'), are being used offshore with some success in a number of locations around the world. In the case of cages near the Hawaiian island of Oahu, the cages remain submerged continuously for aesthetic reasons and to avoid waves.

25.5.6 Alternative culture systems

A number of alternative fish mariculture technologies have been proposed or tested in the past few decades. Most do not appear to be economically or technically feasible at present due in part to the globalization of fish markets that causes intense competition among producers. Some new cage designs are practical and economical, but offer no inherent advantages for dealing with HABs (e.g. surface-oriented off-

shore cages). Alternative systems include floating raceways, onshore tank farms, novel cage designs, and semi-closed systems such as bag culture. Onshore tank farms and recirculating systems (for salmon, for example) are highly risky ventures because they require large capital investments and the culture of fish at extremely high density, where even minor problems can become catastrophes. Onshore tank farms require low elevation but deep-water shoreline areas, and in that respect must compete with ferry and shipping terminals and other competitors for this type of relatively scarce land. Intake volumes of seawater are very high, and no practical means to treat for HABs have been proposed except to pump seawater from very deep waters. While this may be effective, the inability of onshore farms to be economically feasible (except perhaps in Iceland), makes this a moot point.

Enclosed floating pens or bag systems have been developed to maintain a controlled environment for salmon farming (Anon., 1997*a*). The system consists of a flexible, watertight bag enclosure that is held by a flotation collar in the sea. Water can be pumped into the enclosure from any chosen depth to control quality, temperature and avoidance of HABs. The system accommodates oxygen injection, feed-consumption monitoring and waste collection. It is proving valuable in areas subject to surface-oriented HABs such as the Sunshine Coast of British Columbia, which is constantly prone to *Heterosigma* blooms. It may also prove valuable for the production of high-value live-fish markets such as in Hong Kong, where there are recurring anoxia-producing blooms. In some applications of this system, profiling fluorometer data are used to automatically control water intake depth. As fish-killing HABs need not always be in abundance to kill fish, this approach will only work in some areas and with some HAB species. Bag culture does not appear to be economically suited to replace the widespread use of net-pens for normal production of salmonids. In addition, an effective and economical means has yet to be developed to treat and discharge the collected solid wastes that are contaminated with salt from seawater.

25.5.7 Feeding and handling practices

During minor HAB episodes, a useful management practice is to immediately withhold feed at the onset and during the event. This reduces fish digestive demand for oxygen, which is required for other essential metabolic functions. Over prolonged periods of several weeks this practice causes increased physiological stress due to reduced liver glycogen, catabolism of tissues with associated weight loss and an increased susceptibility to chronic diseases such as bacterial kidney disease, all of which may pose economic hardship to the farmer. Cessation of all fish handling and restricted activity on the farm site is a corollary strategy that further limits fish stress by reducing oxygen demand. Unfortunately, these strategies are usually inadequate on their own to deal with major HAB events. Increasing the amount of oxygen-rich carbohydrate in the diet while reducing the oxygen-poor fat content could reduce the metabolic demand for oxygen by the fish. However, carbohydrates are not digested readily by salmonids and the economic feasibility of having carbohydrate-formulated diet for the specific occurrence of a HAB would be untenable.

25.5.8 Water treatment

There has been increasing interest by certain government fishery agencies and the HAB research community in recent years in various means of harmful algal bloom removal by differing types of water treatment. One of the most promising methods

appears to be the use of clay to flocculate cells from the water column. Certain types of naturally occurring clay have been used to treat fish-rearing water to destroy or precipitate HABs in and near fish farms in the Republic of Korea and Japan. Several HAB genera have been treated with apparent success, including *Cochlodinium*, *Chattonella* and *Heterosigma* (Maruyama *et al.*, 1987; Shirota, 1989; Kim, 1997, 1998*a*; Choi *et al.*, 1998). Evaluations of large-scale treatments are by nature very difficult, given the scale of the sampling efforts required.

In the USA, extensive laboratory trials have been conducted to assess the removal efficiency of a wide variety of clay types (Sengco *et al*., 2001). Effectiveness varies greatly among clay types and target HABs, but in most cases is improved by pretreatment with polyaluminum chloride (PAC), a drinking-water pretreatment compound. Studies have begun to assess impacts on cultured fish and the natural environment (Rensel *et al.*, 2000). Excessive use of clay treatment at fish farms has the potential to adversely affect benthic and epibenthic fauna, primarily by sedimentation (burial). Fish farms should not be sited over or adjacent to valuable resources such as shellfish beds, and in the case of salmon farms are increasingly being required to site over erosional bottoms with strong currents. Preliminary experiments have shown some species, such as oysters, to be relatively tolerant to the effects of clay treatment, but others, such as scallops, may reduce feeding rates when exposed to high levels of clay (Ewert *et al*., 2000). Additional research is needed to understand or predict the possible transport of toxin from cells in the water column to benthic sediments and fauna or the induced excystment of cells subjected to clay treatment. Intuitively, those HABs without toxins or with apparently weak toxic effects might be more acceptably mitigated with clay flocculation.

A wide variety of other physical and chemical methods and apparatus has been proposed for HAB mitigation, some with possible merit, but many with high risks of collateral damage to other marine organisms in the treated area (Anderson *et al*., 2001). Many chemicals are capable of killing HAB organisms, but most lack specificity towards target organisms. For example, copper sulfate has been widely used in the past for limiting freshwater blooms of algae, but it is not currently considered a viable option for freshwater or HABs near mariculture because of negative impacts on non-target organisms, contamination of sediments, temporary effects and high costs (Cooke *et al*., 1993). Other chemicals, such as formalin and hydrogen peroxide, entail similar problems and literally hundreds of compounds have been considered or tested, but typically not in well-controlled test conditions.

One large-scale treatment that may be possible is the proposed use of a submerged electrode set at a certain potential to neutralize harmful oxygen radicals produced by *Chattonella antiqua* (Tanaka *et al*., 1994). Also, treatment with sodium per(oxo)carbonate eliminated 90% of *Chattonella* cells after only 2 hours, both in onshore tanks and in the shallow surface layer of Shido Bay, Japan, in 1987 (Okaichi *et al*., 1990). A concentration of 50 mg l^{-1} was required and did not significantly alter ambient pH. A concentration of 0.5 mg l^{-1} sodium hypochlorite, generated by equipment *in situ*, was reported by Kim *et al*. (2000) to effectively remove a number of HAB dinoflagellates. After 2 hours in bright sunlight about half of the initial concentration had been converted to harmless NaCl.

A possible future treatment for aquaculture areas affected by *H. akashiwo* may be the surface addition of freshwater to concentrate cells at the surface for physical removal. Hershberger *et al.* (1997) reported rapid migration of the alga to the surface of columns in the laboratory when very small amounts of distilled water were gently

added to the surface. If a similar procedure is possible in commercial-scale net pens, microalgal cells could be gently skimmed off the surface or treated with clay and replaced with cell-free water pumped from depth.

25.5.9 Therapeutics

At present there are no readily available or government-approved therapeutic drugs designed specifically to treat fish that have been affected by HABs. One of the major killers of farmed fish is considered to be the formation of the superoxide anion radical (O_2^-) by *Chattonella antiqua* and *Heterosigma akashiwo* (Tanaka *et al*., 1994; Oda *et al.*, 1997; Nakamura *et al.*, 1998) and *Cochlodinium polykrikoides* (Kim *et al.*, 1999). The mechanism for death is considered by some to be mucus stripping from the gills of the fish, which leads to osmoregulatory dysfunction and ultimately to death. Reduction of the oxygen radical to the more harmful hydroxy radical is effected in the seawater. Quenching the radicals with the enzymes superoxide dismutase, catalase and glutathione peroxidase has been demonstrated to protect fish in laboratory studies (Yang *et al*., 1995) and could alleviate the problem in affected waters (Colt *et al*., 1991). Addition of such chemicals to the water would depend on the fisheries and environmental agencies in the jurisdiction where the farms are sited, but implementation could hold promise if cost effective.

Physical gill damage from HABs is another major killer of fish (Albright *et al.*, 1992). Consequences of the damage include hypersecretion of mucus and blood hypoxia. Chemicals that reduce mucus production could potentially provide mitigative action. Mucolytic agents fed to fish, such as L-cysteine ethyl ester, have reportedly reduced gill mucus production and sustained fish during exposure to harmful *Chaetoceros* in the laboratory (Yang and Albright, 1994*a*). This appears to be a useful approach to dealing with acute exposures, but extended exposures over months may not be effective for the following reasons. Feeding fish during HABs is not recommended because the high oxygen demand needed for digestion competes with that for basal metabolic maintenance; and moderate amounts of mucus production on the gills from chronic exposure to harmful *Chaetoceros* is likely to be beneficial to the fish. Mucus discharge along with a coughing response provides a defence mechanism for removal of the HABs from the gills (Rensel, 1993). Without mucus, some species of spiny phytoplankton that lodge in the gills would probably be enveloped through lamellar fusion, as has been documented for *Corethron* sp. (Speare *et al*., 1989). Mucus provides a protective barrier and lubricating ability for the gills during stress, without which they are more susceptible to secondary infection from bacteria, viruses and parasites. A different approach using cysteine compounds has been preliminarily tested, not by feeding it to fish, but by treating their culture water (Jenkinson and Arzul, 2001). In these tests, cysteine, ethyl cysteine ester and L-acetyl cysteine were used to reduce 'rheotoxicity' (thickening of the water due to mucus produced by several HABs) and cytotoxity of HABs that included two species of *Gymnodinium*. Some of these chemicals are widely used in medicine as mucolytic agents, which protect by selective reduction of free oxygen radicals. The authors feel that this approach is both technically and economically viable, and should be tested on a pilot scale.

Other drugs such as adrenaline and acetylcholine that are vasoactive agents, regulating the distribution of blood to the gill secondary lamellae (Part *et al*., 1982), could possibly be of some use in treating HAB-affected fish. The need to administer these orally and the potentially high costs, however, limit their usefulness.

25.5.10 Live cage bioassays

Fish farmers should consider placing small, portable fish cages to conduct *in situ* bioassays both at their farm sites and at remote locations where blooms may originate. Small cages have several advantages over large cages normally used in commercial aquaculture. Fish in small cages are more visible for observation of behaviour and easier to sample. They are also exposed to the shallowest waters during surface-oriented dinoflagellate or microflagellate blooms, allowing for a worst-case monitoring of effects. If a suspected HAB is being advected towards a fish-farm site, it may be useful to test the virulence of the harmful species by transporting previously unexposed fish to the bloom and the area of highest HAB concentration. This method has been practised by both researchers and fish farmers for blooms of *Heterosigma akashiwo* and *Cochlodinium* sp. in western Canada and in the Pacific Northwest of the USA (Black *et al.*, 1991; Rensel, unpublished data; Whyte *et al.*, 2001).

25.5.11 Other techniques

Other mitigation practices for HAB-caused fish kills are possible. For some HAB species and nutrient-sensitive areas, these include improvement of the environment by reduction in N and P discharge by municipalities, industry and agriculture. Aeration below the pycnocline to destratify the water column may be useful in some cases if the HAB is dependent on stratified conditions. Pre-emptive fish harvest just prior to a HAB is often practised, but fish should not be sold for human consumption after exposure. This recommendation is not always followed, as many of the HAB species shown in Table 25.1, such as *H. akashiwo*, leave no known toxin or harmful residue in the body tissues consumed.

At least a few species of fish may be acclimated to reduced oxygen conditions that may arise from HAB-caused gill problems. Anaerobic metabolism may be facilitated by increased perfused area of the gills and by elevated oxygen-carrying ability or numbers of erythrocytes in the blood (Heath, 1995; Shepard, 1955). However, acclimation to environmental or blood hypoxia for salmon may not be possible, judging from the results with rainbow trout (Smith and Heath, 1980).

Although not tried on a production scale, large volumes of freshwater could be used to lower the salinity to a level near fish blood content (approximately 10 psu) during a HAB. This would reduce the energetic costs of osmoregulation and slightly increase the oxygen-carrying capacity of water. Bath and Eddy (1979) found that dorsal aorta blood PO_2 of trout in freshwater was about 110 mm Hg. After seawater entry it dropped quickly to 80 mm Hg, then decreased after 28 hours to about 60 mm Hg. This pattern was inversely mirrored by the oxygen-consumption rate. The authors proposed that increased gill osmotic and ionic metabolic activity consumed a significant proportion of the oxygen before it reached the dorsal aorta of the seawater-held fish.

25.6 Overview of fish-kill sampling procedures

25.6.1 Introduction

Fish kills in aquaculture may have a wide variety of causes, including toxic chemical discharges, disease, environmental hypoxia or anoxia due to algal bloom decay, discharge of oxygen-demanding wastes by other industries, etc. In some cases it may be difficult to ascertain the cause of a specific fish kill, as a number of HAB species may be present in water-column samples. An expeditious, broad-scale environmental and fish-tissue sampling campaign is thus needed if the cause is to be revealed. A contingency plan should be prepared by fish farmers to deal with HAB occurrences in a timely manner. A number of literature sources provide detailed information regarding fish kill investigations, including Svobodová *et al*. (1993), Gaines and Taylor (1986) for salmon net-pens, and Meyer and Barclay (1990) for wild fish kills. An overview of the approach and sampling protocols for HAB-caused fish kills in aquaculture is presented below.

25.6.2 Hydrographic and phytoplankton samples

Hydrographic and phytoplankton sampling procedures have been previously discussed. Immediate phytoplankton sampling is perhaps the most important diagnostic tool if a HAB is suspected. It is typically more important than normal techniques of fish-tissue analysis practised by fish pathologists, as many lesions and abnormalities, including those of the gill, are stereotypical of more than one cause (Mallatt, 1985). For example, lifting or separation of gill epithelia is caused by a wide variety of toxicants and chemicals as well as exposure to several HAB species. Phytoplankton samples should be collected from several depths and over a broad enough geographical range to match the movement of tidal waters from the time a fish kill was first observed. If sampling is delayed too long and major weather changes occur or tidal transport is great, the causative HAB species may not be detected. With regard to hydrographic sampling, dissolved oxygen sampling throughout the day, especially just before sunrise, should be conducted if microalgal bloom respiration or upwelling of low-oxygen water is suspected as a cause of fish mortality. Many molecular-based test kits are becoming available for detection of HABs or their toxins (See Chapters 5 and 6). These tests may prove useful for routine sampling and for determining if a bloom is actually toxic, which currently is not always the case (e.g. *Heterosigma akashiwo*).

25.6.3 Gross morphology of affected fish

External appearance of the fish should be carefully noted, including the presence of excessive mucus on the gills or trailing from the opercular cavity, the colour and shape of gills, condition of scales and fins, presence of lesions and other abnormalities. Because of the large volumes of water filtered through fish gills, scraping of the gill surface to prepare a microscope slide as a wet mount can be a simple but effective method to see if certain HAB species are prevalent. This is diagnostic for larger-sized HAB species, such as harmful *Chaetoceros*, which cause gill mucus production and damage. It is much preferred to histological techniques that are time-consuming, expensive and relatively inaccurate due to the extensive tissue-preparation procedures that wash phytoplankton cells from the surface of samples.

25.6.4 Tissue for histopathology, bacteriology and viral analysis

Although the usefulness of fish-tissue analysis may be limited for diagnosing the causative HAB species, as discussed above, an experienced pathologist can provide important insight into fish condition and the possibility of other factors, such as chronic disease, contributing to a fish kill. Sampling of moribund fish and apparently unaffected fish, rather than dead fish, is highly recommended. Fish that have been dead for more than a few minutes may be useless to determine the cause of death using histopathology because tissues are rapidly affected by post-mortem changes (Speare and Ferguson, 1989). For this reason, moribund fish should be dissected and placed in fixative solution immediately. It may be useful to sample the gills, liver, kidney, gut, brain, heart and buccal cavity surfaces to look for aberrations. A variety of stains and counterstains may be used in histology, but alcian-blue stains are particularly useful for highlighting the prevalence of gill or other mucus. As a further precaution, samples for virus and bacterial analyses may be collected to detect the possibility of those causes or contributors to a generalized stress response or death of the fish (Wedemeyer, 1970).

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26

Epidemiology, public health and human diseases associated with harmful marine algae

L. C. Backer, L. E. Fleming, A. D. Rowan and D. G. Baden

26.1 Introduction

A number of human diseases are caused by ingesting seafood contaminated with toxins produced by marine phytoplankton (see reviews by Falconer, 1993; Baden *et al*., 1995; Fleming *et al*., 2001). Phytoplankton are the base of the marine food web, and toxins they produce can accumulate and concentrate in higher organisms. In addition to exposure by seafood ingestion, environmental exposures can occur when marine phytoplankton cells are disrupted by waves as they move onshore. Human exposure to water and aerosols containing toxins and cell fragments during the break-up of Florida red tides have been associated with reports of skin irritation and respiratory distress (Music *et al*., 1973).

The toxins elaborated by marine phytoplankton can be acutely lethal. Although most are primarily neurotoxins, they can cause a wide range of acute and chronic health effects in humans and other species. These toxins are generally tasteless, odourless, and quite heat- and acid-stable; thus normal food preparation methods will not prevent intoxication if the food is contaminated.

In this chapter, the clinical symptoms and epidemiology of human diseases caused by exposure to harmful algae and their toxins are presented, as well as basic epidemiological tools, particularly recommendations about how to investigate an outbreak. A summary of some of the challenges associated with their clinical diagnosis and epidemiological investigation is also provided.

26.2 Human diseases associated with exposure to harmful marine phytoplankton

26.2.1 Overview of diseases and conditions

A number of human diseases are known to be associated with exposure to marine algal toxins (see reviews by Fleming *et al*., 2001; Clark *et al*., 1999; Baden *et al*., 1995; Anderson, 1994; Tester, 1994; Halstead, 1994) Table 26.1 summarizes their characteristics, clinical presentation, geographical distribution, treatment and epidemiology. Diseases associated with marine microalgae result primarily from eating toxin-contaminated seafood (mainly shellfish), and include amnesic shellfish poisoning (ASP), diarrhetic (diarrhoeic) shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP) and paralytic shellfish poisoning (PSP). Other human illnesses

TABLE 26.1 Human diseases and conditions caused by exposure to marine microalgal toxins

Diseases and conditions	Paralytic shellfish poisoning (PSP)	Neurotoxic shellfish poisoning (NSP)	Diarrhetic shellfish poisoning (DSP)	Amnesic shellfish poisoning (ASP)	Ciguatera fish poisoning (CFP)	Aerosolized brevetoxin
Incubation time	5–30 min	30 min–24 hours	<24 hours	<24 hours	<24 hours	Few minutes
Acute symptoms	Diarrhoea, nausea, vomiting leading to paraesthesia of mouth and lips, weakness, dysphasia, dysphonia, respiratory paralysis	Tingling and numbness of lips, tongue and throat; muscular aches; dizziness; reversal of the sensations of hot and cold; diarrhoea and vomiting	Nausea, vomiting, diarrhoea, abdominal pain, chills, headache, fever	Vomiting, diarrhoea, abdominal pain and neurological problems such as confusion, memory loss, disorientation, seizure and coma	2–6 hours: abdominal pain, nausea, vomiting, diarrhoea 3 hours paraesthesia, reversal of hot/cold, pain, weakness 2–5 days: bradycardia, hypotension, increase in T-wave abnormalities	Acute eye irritation, acute respiratory distress (non-productive cough, rhinorrhea)
Chronic symptoms	Unknown	Unknown	Unknown (carcinogen)	Amnesia	Paraesthesia	Unknown
Duration	Days	Days	Days	Years	Months	Unknown
Death rate	1–14%	0%	0%	3%	0.1–12%	Unknown
Route of exposure	Ingestion	Ingestion	Ingestion	Ingestion	Ingestion	Inhalation
Associated foods (transvectors) and/or exposures	Bivalve shellfish, primarily scallops, mussels, clams, oysters, cockles and certain herbivorous fish and crabs	Bivalve shellfish, primarily mussels, oysters, scallops, and other species from the Florida coast and Gulf of Mexico	Bivalve shellfish, primarily scallops, mussels, clams, oysters	Bivalve shellfish, primarily scallops, mussels, clams, oysters and fish	Large reef fish, e.g. grouper, red snapper, amberjack, barracuda (most common)	Aerosolized seawater

…/…

TABLE 26.1 *(Suite)*

Diseases and conditions	**Paralytic shellfish poisoning (PSP)**	**Neurotoxic shellfish poisoning (NSP)**	**Diarrhetic shellfish poisoning (DSP)**	**Amnesic shellfish poisoning (ASP)**	**Ciguatera fish poisoning (CFP)**	**Aerosolized brevetoxin**
Causative organism(s)	Dinoflagellates: *Gymnodinium catenatum*, *Pyrodinium bahamense* var. *compressum*, *Alexandrium* spp.	Dinoflagellate: *Karenia brevis*	Dinoflagellates: *Dinophysis* spp., *Prorocentrum* spp.	Diatoms: *Pseudo–nitzschia* spp.	Epibenthic dinoflagellates: *Gambierdiscus toxicus*, *Ostreopsis* spp.?, *Coolia* spp.?, *Prorocentrum* spp.?	Dinoflagellate: *Karenia brevis*
Treatment	Supportive care Possibly respiratory support	Supportive care (generally self– limiting)	Supportive care (generally self–limiting)	Supportive care (generally self–limiting) Elderly are especially sensitive	IV mannitol Supportive care Tricyclic antidepressants Children more vulnerable	Anti–asthma medications?
Toxin(s) (number)	Saxitoxin (>20)	Brevetoxin (>10)	Okadaic acid, Dinophysistoxins (>6)	Domoic acid (5)	Ciguatoxin (>10), Maitotoxin, Scaritoxin	Brevetoxin (>10)
Toxin molecular mechanism(s)	Na^+ channel blocker	Na^+ channel activator	Phosphorylase phosphatase inhibitor	Glutamate receptor agonist	Na^+-\Ca^{++}-channel activators	Na^+ channel activator
Main endemic geographical area	Temperate areas worldwide	Gulf of Mexico, south-east coast USA, New Zealand	Europe, Japan	East and west coasts of North America	Tropical coral reefs	Gulf of Mexico, south-east coast USA

Sources: Fleming *et al*. (2001), Baden *et al*. (1995), CDC (2001), Music *et al*. (1973).

caused by ingesting toxins from marine microalgae are not necessarily associated with an algal bloom. For example, ciguatera fish poisoning (CFP) results from consuming fish contaminated with ciguatoxins derived from microalgae (e.g. *Gambierdiscus toxicus*) living primarily on coral reefs. Puffer fish (*fugu*) poisoning, is caused by tetrodotoxin, which produces the same physiological effects as PSP toxins, but is more typically associated with bacteria than with microalgae (Kao, 1993).

In addition, direct human exposure to harmful algal blooms (HABs) may result in other health complaints. For example, Music *et al*. (1973) reported acute respiratory distress and eye irritation among people at Palm Beach, Florida, during a bloom of the dinoflagellate *Karenia brevis* (=*Gymnodinium breve, Ptychodiscus brevis*). The symptoms were probably caused by exposure to aerosols containing brevetoxins produced by the dinoflagellate and cellular debris that was driven onshore by the wind.

Although not well documented, the attack rates for these toxin syndromes appear to be high. An attack rate is the proportion of a well-defined population that develops an illness over a specific period of time, where the numerator is the number of new cases that occurred during that period and the denominator is the size of the population at risk (i.e. people who ate contaminated food or breathed air containing an aerosol at the start of the period of interest) (Goodman and Peavy, 1996). For example, in a review of CFP cases in Miami, Florida, Lawrence *et al*. (1980) reported an attack rate of 100% for persons who ate at least one bite of the fish associated with an outbreak.

In areas where seafood is a critical part of a subsistence diet and the toxin-borne diseases are endemic the incidence and prevalence rates can also be high. For example, Dalzell (1994) reported an attack rate for CFP of 10% per year for Niutao Island (population 1,000) in the South Pacific. Morris *et al*. (1982*b*) reported an overall prevalence for CFP in St Thomas, US Virgin Islands, of three cases per 10,000 people per month for 1971, 1977 and 1979.

The populations most likely to be exposed to, and thus affected by, marine algal toxins include: 1) those occupationally involved in seafood harvesting, shipping and processing, 2) seafood consumers, including those consuming seafood they caught or seafood served in a restaurant, 3) environmental workers (especially those collecting samples), 4) individuals who work and play on or near the water, 5) and coastal communities, especially of indigenous peoples who rely on seafood for a substantial proportion of their diet.

26.2.2 Amnesic shellfish poisoning

In 1987, a new type of human toxin syndrome, later termed amnesic shellfish poisoning (ASP), was associated with marine phytoplankton in eastern Canada. In this incident, at least 107 people became ill after eating mussels (Perl *et al*., 1990) which were traced to cultivated stocks in several estuaries in Prince Edward Island. The causative agent, domoic acid, which can act as an excitatory neurotransmitter, was identified both in uneaten mussels served to the victims and in mussels collected from the estuaries. Although extracts of seaweed containing domoic acid have been used in Japan as an ascaricidal agent (Daigo, 1959), no apparent adverse effects of domoic acid consumed in this fashion had been reported in the literature. High concentrations of a diatom, *Pseudo-nitzschia multiseries* (= *Nitzschia pungens*),

identified as the source of the domoic acid in the 1987 poisoning incident, were found in the estuaries used for mussel cultivation.

Victims reported gastrointestinal symptoms (vomiting, abdominal cramps, diarrhoea) and neurological symptoms (incapacitating headache and loss of short-term memory) (Perl *et al*., 1990). Nineteen victims were hospitalised, with seizures, coma, profuse respiratory secretions, or unstable blood pressure, and two or three deaths were blamed at least partially on the contaminated mussels. At least one individual, who consumed the highest dose of domoic acid, became incapacitated by poor short-term memory (Perl *et al*., 1990).

Domoic acid was first identified in California in September, 1991 when pelicans and cormorants were poisoned after eating contaminated anchovies. Domoic acid poisoning was also reported in California sea lions that had eaten contaminated fish (Scholin *et al*., 2000). In razor clams and crabs, domoic acid has been periodically found during routine monitoring by the State Health Departments in Oregon and Washington, and 25 cases of ASP have been associated with consuming toxic razor clams (Washington Department of Health, 1994). Routine monitoring and closing of contaminated shellfish beds have undoubtedly prevented other cases of ASP in Canada. Domoic acid is often found at low levels in shellfish from Atlantic Canada and in other regions throughout the world, and extensive harvest closures were imposed in the spring of 2002 in eastern Canada because of unusually high concentrations of domoic acid in shellfish tissues.

26.2.3 Diarrhetic shellfish poisoning

The first confirmed incidence of human gastrointestinal illness associated with consuming mussels exposed to dinoflagellates occurred in the Netherlands in 1979 (Kat, 1979). Similar incidents occurred in Japan in the late 1970s (Yasumoto *et al*., 1978). This syndrome, called diarrhetic shellfish poisoning (DSP) – a misnomer for the more accurate term *diarrhoeic shellfish poisoning* – appears to be directly related to the consumption of contaminated bivalve shellfish, particularly mussels and whole scallops.

This toxin syndrome is still primarily found in Europe and Japan, but outbreaks can involve hundreds of people (Aune and Yndestad, 1993). Cases have been sporadically reported from Chile and Nova Scotia, as well as Australia, Indonesia and New Zealand (Sundström *et al*., 1990). In addition, DSP toxins have been identified in shellfish from a wide variety of other regions, including India, the former Soviet Union, Uruguay and Long Island, New York, suggesting that outbreaks may also be occurring, but perhaps not reported, in these areas (Aune and Yndestad, 1993).

Several toxins are associated with DSP: these include okadaic acid and dinophysistoxins (DTXs), particularly DTX1 and DXT3, but there are also other congeners with poorly defined toxicity and diarrhoeagenic properties (see Chapter 8). These toxins are produced by dinoflagellates that may be either planktonic (e.g. *Dinophysis* spp.) or primarily epi-benthic (e.g. *Prorocentrum* spp.). Mussels and scallops feeding on these microalgae accumulate the toxins, particularly in the hepatopancreas (Aune and Yndestad, 1993) and people are exposed when they eat the shellfish. The minimum doses estimated to cause diarrhoea in adults are 40 µg for okadaic acid and 36 µg for DTX1 (Aune and Yndestad, 1993).

The symptoms of DSP are gastrointestinal (diarrhoea, nausea, vomiting, abdominal pain) and occur within 30 min to 3 hours of ingestion of contaminated

shellfish. People affected by DSP feel very sick, but they reportedly recover completely within a few days (Kat, 1979; Yasumoto *et al*., 1978). Although the acute disease is not life-threatening, *Dinophysis* spp. and *Prorocentrum* spp. produce additional toxins that have hepatotoxic, immuno-suppressive and tumour-promoting activities in animals (Aune and Yndestad, 1993). Studies of chronic health effects in people with histories of DSP have not been conducted. However, evidence exists in both animals and humans that exposure to DSP toxins may be a risk factor for cancer. For example, there have been reports (Landsberg, 1996; Van Beneden, 1997; Cordier *et al*., 2000) of increasing incidence of gonadal tumours in shellfish exposed to carcinogenic marine phytoplankton toxins. The long-term health of people who consume these potentially carcinogenic toxins is an important component of their potential public health impact.

26.2.4 Neurotoxic shellfish poisoning

Neurotoxic shellfish poisoning (NSP) has been known since the early 1990s in the south-eastern USA (primarily along the Gulf coast) and eastern Mexico (Steidinger, 1993). There is also evidence of similar events associated with human consumption of shellfish from New Zealand (Ishida *et al*., 1996). Outbreaks of NSP have involved toxic oysters, clams and other suspension-feeders that accumulate toxins during red-tide events. Bay scallops may also accumulate toxins, but people usually eat only the adductor muscle (where the toxins are considerably less concentrated) and not, as with certain other bivalve shellfish, all the soft tissues. The incidence of NSP is very low in the south-eastern USA because of routine monitoring programmes for both the causative organism in coastal waters and for the toxins in seafood, prompting closures when shellfish become toxic.

The toxins associated with NSP (see Chapter 8) are a group of polyether compounds called brevetoxins (Baden, 1989; Schulman *et al*., 1990). Although several gymnodinoid dinoflagellates produce toxins that are capable of killing fish, *Karenia brevis* is rather unusual in that it produces the brevetoxins associated with neurotoxic shellfish poisoning.

Both brevetoxins and ciguatoxins are ladder-frame polyethers that bind to the same receptor site on the sodium channel. The acute symptoms of NSP are similar to those of CFP and include abdominal pain, nausea, diarrhoea, burning pain in the rectum, headache, bradycardia and dilated pupils. Additional reported symptoms include temperature-sensation reversals, myalgia, vertigo and ataxia (Hughes and Merson, 1976; Sakamoto *et al*., 1987; McFarren *et al*., 1965; Baden, 1983). Aerosolized brevetoxins may also induce respiratory distress and eye irritation in those exposed to the toxins (Music *et al*., 1973).

Brevetoxins were implicated in the deaths of manatees in Florida (O'Shea *et al*., 1991) during a widespread bloom of *Karenia brevis*. At necropsy, the animals did not appear to be unhealthy and they had recently fed. Elimination of many alternative explanations (e.g. infections, injuries) led to the hypothesis that the deaths resulted from exposure to this organism at high concentrations.

26.2.5 Paralytic shellfish poisoning

Paralytic shellfish poisoning (PSP) has been known to native Americans of the Pacific Northwest for centuries (Kao, 1993). Early human intoxications have been recorded mostly in North America and Europe (Prakash *et al*., 1971), but incidents

have been reported elsewhere, including Malaysia, the Philippines, Indonesia, Venezuela, Guatemala (Rosales-Loessener *et al*., 1989; Kao, 1993), China (Anderson *et al*., 1996) and South Africa (Popkiss *et al*., 1979). This toxin syndrome occurs in humans after ingesting bivalve shellfish (clams, mussels, scallops, etc.) contaminated with one or more of a group of structurally related congeners of saxitoxin, which accumulate in the soft tissues, particularly in the hepatopancreas (digestive gland) of these suspension-feeding organisms (Halstead and Shantz, 1984). Ingestion of coral reef crabs and gastropods (e.g. moon snails) has also been known to cause PSP in Japan and Fiji (Noguchi, 1969), and one report from Indonesia involved finfish as the transvector (Adnan, 1984).

The PSP toxins are produced by dinoflagellates of the genera *Gymnodinium* (Anderson *et al*., 1989), *Alexandrium* (Anderson *et al*., 1996), and *Pyrodinium* (Halstead and Shantz, 1984). The biological activity of these toxins is based on selective blockage of the voltage-gated sodium channel of many excitable membranes, thus blocking the generation and propagation of action potentials in nerve axons and skeletal muscle fibres (Kao, 1993). Humans and other mammals, as well as birds and fish, can be affected by PSP toxins. Humans are very sensitive to PSP toxins; saxitoxin is the most potent analogue – the fatal oral dose is 1–4 mg – depending on the age and physical condition of the victim (Baden *et al*., 1995).

The onset of PSP symptoms usually occurs within 30 min to about 3 hours ; the severity of the symptoms depends on the amount of toxin ingested and absorbed by the body. Children are apparently more severely affected than adults (e.g. Roy, 1977; Rodrigue *et al*., 1990) because they are more sensitive to saxitoxin, or they may metabolize the less-potent sulfamate congeners (toxins C1, C2, B1, B2) to more potent carbamate derivatives more rapidly than adults (Rodrigue *et al*., 1990). The initial symptoms of PSP are paraesthesia (tingling) and numbness around the lips and mouth (see Kao, 1993). These sensations then spread to the face and neck. In addition to the neurological symptoms, victims may experience nausea and vomiting. In moderately severe poisonings, paraesthesia progresses to the arms and legs, which also exhibit motor weakness. Victims may experience dizziness, incoherent speech and light-headedness. Respiratory difficulties may begin with a feeling of tightness around the throat. In severe poisonings, muscular paralysis spreads and becomes deeper. Death can result from decreasing ventilatory efficiency and increasing hypoxia. Victims typically do not experience hypotension or pulse abnormalities. The half-life of PSP toxins is short (90 min), making supportive management, especially hospitalization and ventilatory support, effective in treating acute PSP.

The human death rate from PSP varies considerably, from no deaths in recent outbreaks in the USA and Europe to rates of 2–14% in other parts of the world (Kao, 1993). The frequency of mortalities is primarily related to the availability of emergency hospital care and past experience with PSP outbreaks.

The incidence of PSP appears to be increasing. Before 1970, 1,600 cases of human intoxication had been reported. Since that time, however, 900 additional cases have been diagnosed (Kao, 1993). The effects of intoxication by PSP toxins are so characteristic that it is unlikely that this increase in cases is due only to better reporting.

26.2.6 Ciguatera fish poisoning

Ciguatera fish poisoning (CFP) outbreaks typically occur in a circumglobal belt extending approximately from latitude 35° N. to 34° S. (Hessel *et al*., 1960), which includes Hawaii (Hokama *et al*., 1993), the South Pacific (Bagnis *et al*., 1979), the Caribbean and Indo-Pacific (Lange, 1987) and the US Virgin Islands (Morris *et al*., 1982*b*) (see also Chapter 10). Ragelis (1984) estimated that 50,000 to 500,000 cases occur each year in the islands of the Pacific Ocean and Caribbean Sea. In the USA, CFP is the most common food-borne illness caused by a marine biotoxin. Although most cases occur in Hawaii and southern Florida, or after travel to the Caribbean, other cases have occurred in non-endemic areas when people ate fish caught in endemic areas (Glaziou and Legrand, 1994).

The unicellular marine dinoflagellate, *Gambierdiscus toxicus*, primarily associated with coral reefs (Legrand *et al*., 1992), is the origin of ciguatoxin and congeners, a group of lipid-soluble polyether compounds. The CFP toxins, which also include more hydrophilic components called maitotoxins, are passed through the coral reef food web to large fish (both herbivores and carnivores) and accumulate in fish tissues (Lange, 1987). The most common fish associated with CFP are groupers (e.g. *Plectropoma* spp.), barracudas (*Sphyraena* spp.), hogfish, snappers (e.g. *Lutjanus bohar*), carrang (*Caranx ruber*), old wife (*Balistes vetula*), kingfish, parrotfish and small surgeon fish (Dalzell, 1994; Escalona de Motta *et al*., 1986; Morris *et al*., 1982*b*). When caught, the fish appear to be healthy and have a normal taste and appearance (Lange, 1987). In addition, different fish accumulate different amounts of toxin, and the toxicity of one individual does not necessarily predict the toxicity of other fish caught in the same geographical area.

Ciguatera fish poisoning is characterized clinically by early gastrointestinal effects (appearing a few hours after eating the fish) that are accompanied or followed by neurological and occasionally by cardiovascular symptoms (e.g. bradycardia, hypotension) (Glaziou and Legrand, 1994). Sensory disturbances, such as paraesthesia of the perioral region and distal extremities (i.e. numbness of the mouth and extremities), generalized pruritis (rash) and reversal of temperature sensation are distinctive features of the disease. Other commonly reported symptoms include arthralgia, metallic taste and dizziness (Escalona de Motta *et al*., 1986; Morris *et al*., 1982*b*) and general weakness (Quod and Turquet, 1996). The gastrointestinal symptoms usually persist for only a few days, whereas the neurological symptoms may persist for up to several months (Quod and Turquet, 1996; Blythe *et al*., 1994; Glaziou and Legrand, 1994). In the Pacific, chronic neurological symptoms have been reported up to 25 years after ingesting the toxin.

Symptoms of CFP are more common in people suffering from a second or subsequent poisoning, perhaps because of an accumulation of the toxins in their bodies, and may be more severe in people who eat carnivorous, rather than herbivorous, fish (Bagnis *et al*., 1979; Glaziou and Martin, 1993). Also, symptoms have been reported to recur when fish of any type, alcohol, caffeine or nuts was ingested within 3 to 6 months of initial exposure (Baden *et al*., 1995). Finally, men appear to be at higher risk of poisoning, whatever the severity; however, it is not unusual for a Polynesian fisherman to eat part of a fish to make sure it is not toxic before sharing it with the rest of his family (Glaziou and Martin, 1993).

As indicated above, CFP is pleiomorphic and many of the symptoms are subjective, making this disease one of the most challenging to diagnose (Pearn,

1994). In addition, the time frame for effective treatment (i.e. supportive therapy and intravenous mannitol soon after exposure: Bagnis *et al*., 1991; Blythe *et al*., 2000) makes rapid diagnosis essential. Overlooking the possibility of CFP rather than documenting the symptoms and signs remains the major problem in managing cases, particularly in areas where it occurs only sporadically (Pearn, 1994).

26.3 Epidemiological tools

26.3.1 Planned epidemiological research

Epidemiology is the study of the distribution and determinants of disease in populations and the application of the results to the control of health problems (Last, 1988). To understand an epidemiological problem, an investigator must develop an understanding of the characteristics of the host (i.e. age, sex, race, occupation and predisposition to disease), the agent (i.e. biological, physical and chemical properties) and the environment (i.e. physical, biological, social, and economic factors) (Last, 1988). Epidemiological research, including case-control studies and cohort studies, are critical in determining the interaction among host, agent and environment, and planned epidemiological studies are useful in evaluating many aspects of human illness associated with HABs.

26.3.2 Public health surveillance

Disease surveillance is an important epidemiological and public health tool. Public health surveillance involves the collection, analysis and dissemination of data on specific health effects (e.g. disease, disability) for use in public health practice (Thacker and Berkelman, 1998). A surveillance system includes the capacity to collect and analyse data, as well as the ability to disseminate the data in a timely manner to public health agencies that can then undertake effective prevention and control activities.

Surveillance can be established to assess public health status, establish public health priorities, evaluate programmes and conduct research (Teutsch, 1994). Surveillance data can be used to estimate the magnitude of a health problem, inform the population at risk of exposure or illness, inform persons and organizations responsible for immediate control measures and other interventions, understand the natural history of an illness or injury, and detect outbreaks or epidemics. Surveillance data can also be used to document the distribution and spread of a health event, test hypotheses about etiology, evaluate control strategies, monitor changes in exposure, identify research needs, assist epidemiological and laboratory research, facilitate planning and provide information for decision-making and policy (Teutsch, 1994).

Surveillance systems collecting primary data are classified as either passive or active (Teutsch, 1994). A passive system receives voluntary reports from physicians, individuals or institutions. An active system regularly contacts previously identified sources to elicit reports. An active system results in more complete reporting than does a passive system; however, active surveillance is labour-intensive and costly.

Disease reporting can facilitate the tracking of an outbreak and perhaps even identify the sentinel case. Surveillance systems prospectively identify the particular

outcome of interest. Most surveillance systems also collect at least limited demographic information about the affected people, details about the health event, and the presence or absence of the appropriate risk factors (Klaucke, 1994).

In the USA, requirements for reporting diseases and conditions are mandated by state and territorial laws and/or regulations (CDC, 2001). The Council of State and Territorial Epidemiologists (CSTE) and Centers for Disease Control and Prevention (CDC) collaborate to determine which diseases are nationally notifiable (i.e. must be reported to the CDC). Although physicians are required by law to report a number of food-borne diseases and conditions that they encounter, they are not required to report those associated with exposure to marine microalgae or their toxins. However, some state health agencies require that physicians and/or diagnostic laboratories report such cases to a health agency. For example, Florida requires physicians to report CFP and NSP to the state health department (Florida Department of Health, 2001). In Alaska, PSP is considered a public health emergency requiring immediate reporting to the state health department (Alaska Department of Health and Human Services, 2002).

An example of surveillance for newly emerging public health issues is the Possible Estuary-Associated Syndrome (PEAS) surveillance system (CDC, 1999). In autumn 1997, reports of fish kills and human illnesses possibly associated with exposure to the marine dinoflagellate *Pfiesteria piscicida* and/or *Pfiesteria*-like organisms generated enormous media attention and public health concern on the eastern coast of the USA. It was unclear whether the reported biological effects involved exposure to the organism itself, any toxins it might produce, or even symbiotic organisms such as bacteria. The CDC developed exposure and symptom criteria that could be used to evaluate the extent of this public health issue (CDC, 1997). Using exposure to estuarine water as an indicator of potential exposure, the PEAS surveillance system was originally developed to evaluate the public health burden associated with state health agency responses to questions about, and human exposures to *P. piscicida* and similar organisms. The system was implemented on 1 June 1998 and as of 31 January 2001, six states (Delaware, Florida, North Carolina, Maryland, South Carolina and Virginia) received 3,333 phone calls about *Pfiesteria* and PEAS. Although only five individuals have met the exposure and symptom criteria for PEAS (Backer *et al*., 2001), the public health activities conducted in association with PEAS surveillance provided both information and a methodology that can be applied to other HAB-related illnesses.

26.3.3 Field epidemiology

26.3.3.1 *Overview of field epidemiology*

Although planned epidemiological studies and surveillance are important in evaluating human illnesses associated with exposure to HABs and their toxins, the typical response to a report of a food-borne illness or environmental exposure is a field (or outbreak) investigation. The unique issues that must be addressed during field investigations constitute a discipline in itself (Goodman and Buehler, 1996). For example, in contrast to planned epidemiological studies, toxicity outbreaks associated with microalgae are typically unexpected, and may require both an immediate public health response, and that epidemiologists and other public health officials travel to the field. Often, the extent of the investigation is limited because of the need for timely intervention. Field epidemiologists must decide when the data are sufficient

to take action rather than examining what questions the data can answer (Goodman and Buehler, 1996).

There are challenges associated with conducting field investigations (Goodman and Buehler, 1996). Data for field investigations are obtained from a variety of sources, including medical records and anecdotal reports, which vary in usefulness, completeness and accuracy. In contrast to planned epidemiological studies with calculated sample sizes, outbreak investigations typically involve a small number of cases, thus limiting both the study design and statistical power. Assessing exposure in the field may also be difficult because field investigations begin after the onset of symptoms, so suspected food items may have been consumed, or the source of an environmental exposure (such as a HAB) may have disappeared.

Field epidemiologists face additional challenges. Outbreaks of human illness may attract local media attention, which can directly affect the investigation. They may be linked, appropriately or not, to specific industries, restaurant chains or recreational activities; and people may be reluctant to participate in an investigation if they believe it could affect their livelihood. For example, since 1967 on the islands of Réunion and Mauritius in the Indian Ocean, harvesting or importing certain species of commercially valuable fish has been illegal because they may be contaminated with ciguatoxins. However, incidents of CFP still occur in these areas (Quod and Turquet, 1996) and field investigations may be hampered by the reluctance of victims to reveal the consumption of illegally caught fish.

In areas where CFP is endemic, outbreaks may actually be unremarkable and thus difficult to assess. In the South Pacific, where near-shore and coastal fish are a major source of animal protein (Dalzell, 1994), people normally avoid fish potentially contaminated with ciguatera toxins, but readily consume it when alternative food sources are unavailable. The community accepts the risk of poisoning, and although CFP is the eighth most commonly reported illness in the area, it is not considered an important public health problem in these island countries (Dalzell, 1994).

Despite these challenges, the nature of illnesses caused by marine algae makes rapid response critical. Fortunately, recommended methods for conducting field epidemiology activities directly apply to investigations into them.

26.3.3.2 *Conducting field investigations*

Although most epidemics involve acute infectious agents, the same epidemiological and public health principles apply to investigations of non-infectious diseases. Gregg (1996) described the 10 basic steps for conducting a field investigation (Table 26.2). Several of these tasks can be done simultaneously or in a different order. For example, the final step listed in the table is to execute control and prevention measures. However, with PSP events, the appropriate control and prevention measures, i.e. verifying where the shellfish came from and possibly closing those shellfish beds for harvesting until they are no longer toxic, should be initiated as soon as a case is diagnosed.

Often, a field investigation begins after an epidemic has subsided and most of the information collected will be based on memory. Health officials, physicians and patients may all recall different aspects about the event, or for various reasons, it may not be accurately reported to the investigator. The epidemiologist may have to rely on supposition without the benefits of clinical tests to verify the information.

Local health officials typically will know whether cases of a particular disease are higher than normal. However, if it has not been previously identified in the area, or has a clinical description that differs from the typical signs and symptoms, they may be unaware of the outbreak. In this instance, a survey of physicians, emergency departments, poison information centres or community households may strongly support the existence of an epidemic.

In a typical field investigation, the epidemiologist will be able to confirm a clinical diagnosis with standard laboratory tests. However, diagnosis of the symptoms caused by marine microalgae relies more heavily on a history of the patient's diet or environmental exposure, including routine monitoring data, than on results from clinical tests.

The next steps in a field investigation are to create a working case definition, decide how to find and enumerate cases. Existing criteria may be available for developing a case definition (e.g. gastrointestinal complaints occurring simultaneously with, or preceding, neurological symptoms for a presumed ciguatera outbreak), or a new group of symptoms may have to be defined. Most importantly, the case definition must be applied in exactly the same manner to all people under investigation.

The sensitivity (the number of people ill who are identified as cases) and specificity (the number of people not ill and not identified as cases) (see Hennekens and Buring, 1987) of the case definition determine how many cases might be missed and how many non-cases might be included. The sensitivity and specificity of a case definition can be determined by how the number of people involved in the outbreak compares with the usual number of cases identified in area clinics, whether clinical signs and symptoms exist, the availability and accuracy of clinical or laboratory tests for verification and the accessibility of case patients.

Case-finding methods will vary depending on the outbreak setting and the disease. Soliciting information directly from physicians, hospitals and community members will help to identify cases. Although the methods will vary, the epidemiologist must establish a system for case-finding that can be used during the investigation and afterwards.

TABLE 26.2 The 10 steps of an epidemiological field investigation

1. Determine the existence of an outbreak.
2. Confirm the diagnosis.
3. Define a case and count cases.
4. Orient the data in terms of time, place and person.
5. Determine who is at risk of becoming ill.
6. Develop a hypothesis explaining the specific exposure that causes disease and test this hypothesis by appropriate statistical methods.
7. Compare the hypothesis with the established facts.
8. Plan a more systematic study.
9. Prepare a written report.
10. Execute control and prevention measures.

Source: Gregg, (1996).

Of course, identifying cases is not enough. Controlling and preventing disease depends on knowing the source of the outbreak and the method of exposure to the causative agent. Information that might provide clues about the origin and natural history of the epidemic can be collected when patients are interviewed. The first step is to collect basic demographic information, including age, sex, residence and occupation. The next step is to collect information about the illness, including date of onset, signs, symptoms and laboratory test results. If the disease is potentially water or food-borne, then the investigator should identify the patient's history of water and food consumption prior to the onset of symptoms. If transmission is by personal contact, then information about the frequency, duration and nature of personal contacts should be collected. If the etiology is unknown, then a wide range of information covering the possible risks and routes of disease transmission should be identified.

When multiple cases have been identified, and as early in the investigation as possible, the disease can be characterized in terms of person, place and time. Identifying why an outbreak occurred will allow more pertinent data collection. For example, an epidemic is characterized in time using an epidemic curve, which is a histogram plot of the time of illness onset (*x*-axis) and the number of cases (*y*-axis). The epidemic curve can provide information about the magnitude of the outbreak, the mode of spread and the possible duration of the epidemic.

As an example, the epidemic curve in Fig. 26.1 shows an outbreak of PSP in Papua New Guinea (Rhodes *et al*., 1975). This curve could possibly represent two separate events, each involving a simultaneous common exposure of many people. It

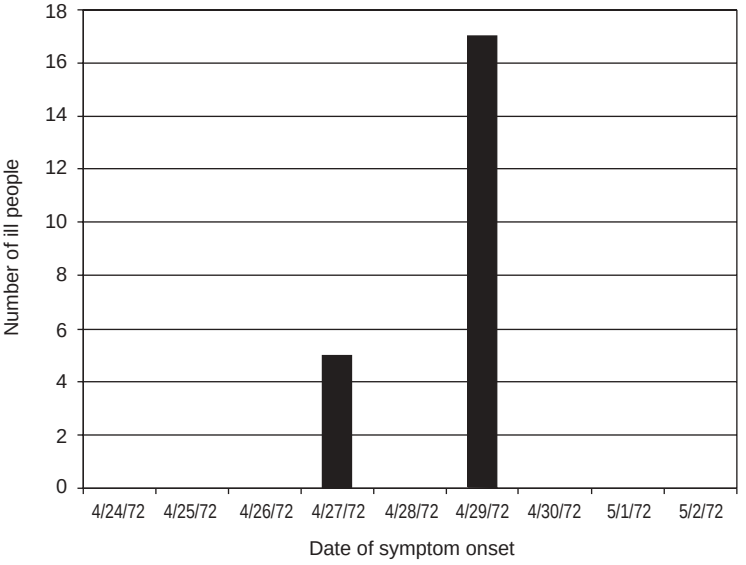

Figure 26.1
Epidemic curve for an outbreak of paralytic shellfish poisoning (PSP) in Papua New Guinea. The dates of the outbreak were 26–29 April 1972. The histogram shows the number of people who became ill on each day. *Source:* Rhodes et al. (1975).

also suggests a short incubation period and the elimination of exposure shortly after the outbreak began. Based on the report by Rhodes *et al*. (1975), on Wednesday 26 April 1972, a woman and her four children had a meal of boiled shellfish they had harvested from the beach in Walai, a Papuan coastal village. Early on Thursday morning, the family members became ill and the youngest child died before dawn. On Thursday, no shellfish were gathered from the beach because of a mourning service that included a meal of tinned fish and other foods. On Friday, more shellfish were harvested from the beach and were eaten by people living on the east side of the village (38 people) but not by those on the west side (19 people). All the people on the east side ate at least some shellfish, 19 became ill and one person died. After the second incident the villagers reportedly abandoned the village and the outbreak was over.

An epidemic can occur in a unique location in the community and if this can be illustrated, it may provide clues about the source or route of exposure. Geographical plots may provide a distribution map that closely follows sources and routes of exposure, such as water-distribution systems, sewage-disposal outflows, prevailing-wind currents, air-flow patterns in buildings, recent recreational activities and habitats of transvectors, helping to identify people at risk.

Lastly, the number of human characteristics that can potentially affect an individual's response to a given exposure are virtually limitless and may include sex, race, nutritional status or even specific polymorphisms in the enzymes that metabolize toxins.

When the field investigation team understands how many people are ill, when and where they were when they became ill, and what their characteristics are, it will generally have either a firm or a good 'working' diagnosis of the disease and can identify people at risk. For example, a person, place and time description of an epidemic might strongly suggest that only people who ate oysters harvested in a particular Florida bay during a red-tide bloom were at risk of getting NSP. However, if an epidemic is widely distributed or involves people of many age groups with no obvious common characteristics, then the team may have to conduct a survey to obtain more specific information about the people who are sick before it can identify who is at risk.

Once the epidemic has been described in terms of person, place and time, the exposure is identified by developing and testing a hypothesis using appropriate statistical methods. Histories of exposure are obtained from all persons who became ill and compared with those of people who did not become ill. If they are significantly different a new hypothesis must be developed.

For example, suppose a field investigation involved an outbreak of nausea, vomiting and diarrhoea among 22 people aboard a cruise ship. A survey of the ill passengers indicated that they all became ill within 2 hours of eating at a dinner buffet. Initially it was hypothesized that the victims had been exposed to food contaminated with a microbial or chemical agent. People who ate at the buffet were identified and interviewed to determine what foods and beverages they had taken. Early analysis showed that all but two of the ill people ate steamed mussels. This would indicate how popular mussels were among those who were ill but would not provide enough evidence to support the hypothesis they were the causative agent.

The next step would be to compare mussel consumption of the ill passengers with mussel consumption of passengers who ate at the buffet but who did not become ill. In this hypothetical example the food histories were similar between the

two groups, except for mussel consumption. Although 20 of the 22 (91%) ill passengers reported eating the mussels, only 33% of passengers who ate the mussels did not become ill. A comparison of the two groups using the statistical software Epi Info 6.03 (Dean *et al*., 1995) gives a Chi-square value of 24.61. Such a difference in rates would occur by chance less than once per 100,000 if eating mussels was not related to becoming ill (i.e. the probability, *p*, of this association occurring by chance is <0.000 001). This statistical evidence can be supported by chemical analysis of the leftover food. For example, if okadaic acid were identified in samples of the mussels and they were implicated as the source of exposure in this outbreak, the syndrome would be identified as DSP.

Once the investigating team identifies the most probable method of exposure, the epidemic characteristics must be compared with the known facts of the disease. For example, if the investigation identified drinking water as the most likely common source of exposure, then marine algal toxins would be an unlikely origin.

When the field investigation is complete and the data have been analysed, the investigators may need to identify additional cases to improve the definition of the epidemic or to evaluate a new laboratory method. For example, if a new method has been developed to measure one of the toxins in human serum, then requesting blood samples from study participants might be useful. Additional questions refining previous food-consumption histories and symptoms may be useful in identifying a dose-response.

Usually, a field team's final responsibility is to prepare a written report documenting all aspects of the investigation. The report should be written as soon as possible because it may provide the impetus or justification for control and prevention efforts. A record of the time and resources expended in the investigation will help to document the magnitude of the health problem and may justify the development of a programme, such as disease surveillance or improved seafood monitoring. Finally, a well-written report describing a field investigation is an important instrument for alerting the public health community to potential sources, geographical distributions and populations at risk of marine algal-associated illnesses.

26.3.3.3 *Operational aspects of an epidemiological investigation*

The operational aspects of epidemiological investigations are presented in Goodman *et al*. (1996) and are summarized in this section. Investigation of disease outbreaks involves the co-operation of investigators from a number of disciplines including clinical medicine, epidemiology, analytical and clinical chemistry and environmental health. Co-operation from the local seafood and tourist industries may also be critical to investigations of marine toxin-related outbreaks. The investigators must work together to identify the important features of the outbreak, including person (Who is ill? Who is not? What are the symptoms?), place (Where did the exposure occur?) and time (What is the temporal association between the exposure and the outcome?). A rapid response following identification of an outbreak may identify the exposure and prevent more cases.

A primary consideration for field investigation is a formal request for assistance from an authorized official. In the USA, the authority is with the state and local health agencies. A state or territorial epidemiologist or a local public health official will decide whether to investigate an outbreak or to seek assistance. In other countries, or with a multinational organization such as the World Health Organization, a minister of health may have such authority.

A decision to conduct field investigations will be based on one or more of the following objectives:

(a) to control and prevent further disease;
(b) to provide additional information about the interactions among the human host, the disease agent and the environment;
(c) to evaluate the quality of surveillance at the local level or to determine the need to establish surveillance; and
(d) to provide training opportunities in field epidemiology.

If field assistance is requested, a number of issues should be discussed with the visiting team. For example, the investigating team needs to know which resources are locally available and which they need to provide, as well as who will direct the day-to-day investigation and provide overall supervision. Finally, those involved in requesting and conducting the investigation need to agree on how the data will be shared, who will write the report, and who will be senior author of any scientific papers describing the investigation.

Investigators frequently seek advice on statistical methods and consult with other experts (e.g. veterinarians, taxonomists, environmental experts). Consulting with laboratory experts before embarking on an investigation will ensure that the proper specimens, if any, are appropriately collected, stored, shipped and analysed. In addition, an information specialist who can respond to public inquiries and co-ordinate contacts with the media may be an important component of the team.

Field investigations can be complex, and because circumstances during the investigation can be distracting, preparing a list of tasks may be useful to ensure its orderly progression. The mnemonic SLACK OFF, developed in 1986 by Jeffrey Sacks (Goodman *et al*., 1996), encompasses the tasks needed to organize and manage the activities of a field investigation (Table 26.3).

TABLE 26.3 The SLACK OFF framework for field investigations

Mnemonic	**Definition**	**Activity**
S	Shells (table shells)	Create 2×2 table shells needed to answer questions about the epidemic. Collect sufficient data to classify or stratify levels of exposure.
L	Log decisions	Record decisions as they are made.
A	Accuracy	Remember the need for quality control, including training and monitoring data collectors and abstractors, conducting error checks, validating data and evaluating non-respondents.
C	Communication	Be responsive to the need for external (e.g. the press) and internal (e.g. within the team) communication.
K	KISS (keep it simple)	Try to reduce the problem to one 2×2 table.
O	Ongoing writing	Write down why you are there; write while the investigation is ongoing; write down methods as you define them.
F	Filing	Maintain an inventory of data files.
F	Friendship	Make a special effort to maintain morale.

Source: Goodman *et al*. (1996).

26.4 EPIDEMIOLOGICAL DATA COLLECTION AND ANALYSIS

In this section, suitable methods for the collection and description of epidemiological data to test a specific hypothesis and calculate the measures of association used to describe the relation between exposure and adverse health outcome are summarized. Many resources are available that describe the statistical analysis of epidemiological data, therefore this issue is not addressed here in detail.

26.4.1 Planning epidemiological data analyses

Analysis of data collected during an epidemiological field investigation should be considered during planning of the investigation (Dicker, 1996). By talking with patients, health-care professionals and others, investigators can generate one or more hypotheses to describe the observed illnesses. Then the team can design the appropriate methods (e.g. questionnaire, medical records abstraction forms) to collect the data needed for hypothesis testing. The information collected should adequately describe person, place and time to allow comparison among groups and to distinguish case-patients from control individuals.

Once the data are collected, the investigators should develop an analysis strategy or guide to follow during the data analysis, such as the following, described by Dicker (1996).

First, the investigators should examine how the data were collected. For example, if the data were collected using a case-control study design, the strategy should include comparing cases with controls. If the study was a cohort study, it should include a comparison of exposure groups.

Second, the investigators should decide which variables such as exposure, outcome, or known risk factors, are the most important. They should look carefully at the frequency of responses and descriptive statistics (e.g. minimum, maximum, mean, mode) for each of these variables.

Next, table 'shells' should be sketched out, including frequency distributions and two-way tables that are completely labelled but contain no data. A useful series of five table shells suggested by Dicker (1996) includes a table of clinical features of this illness, a table of descriptive epidemiology, a table of primary associations, (i.e. risk factors by outcome status), a table stratifying the primary associations to assess confounding and effect modification and effect modification and specific subgroup analyses. The table shells, which guide data analysis, will be filled in as the analysis progresses.

In many epidemiological investigations, the exposure and outcome of interest can be characterized as binary variables, i.e. 'yes' or 'no', and the relation can be characterized using a two-by-two table (Table 26.4) (Dicker, 1996). One convention is to label the table across the top with the disease status (well versus ill), and along the side with the exposure status (exposed versus unexposed). The intersection of a row and a column is called a cell, and the cells are denoted a, b, c and d. The row and column totals are provided, and the total number of people included in the two-by-two table is written in the lower right corner. The attack rates may be provided to the right of the row totals.

26.4.2 Describing epidemiological data

Descriptive epidemiology attempts to identify the frequency of a disease as well as the time, place and population affected (Goodman and Peavy, 1996). The easiest way to examine the extent of a disease is to enumerate the number of cases and compare with historical records while searching for unusual patterns or outbreaks. These case counts must then be evaluated in the appropriate perspective to ascertain if this is a new outbreak or if changing methods have resulted in an increase in the number of cases reported.

The number of cases also needs to be compared with the number of people in the population in which the cases occurred. Rates are measures for relating the number of cases to the population. Death rates describe deaths in a population over a given period and morbidity rates describe the frequency of an illness in a population over time.

Other measures (i.e. incidence, attack rate and prevalence) are also used in epidemiology to quantify the amount of disease in a population (Goodman and Peavy, 1996) and are briefly described here. Incidence measures the number of new cases in a population over a specific period. The numerator is the number of new cases and the denominator is the size of the population. Incidence is most useful for describing acute illnesses, such as the number of new cases of DSP diagnosed in a specific population in a specific year.

An attack rate is the proportion of a population that develops an illness over a specific period. The numerator is the number of new cases during that time and the denominator is the size of the population at risk at the beginning of the time. In our earlier example of DSP illnesses among cruise ship passengers, the attack rate was 20/53 (the number of ill people/the number of people who ate mussels) or 38%.

Prevalence measures the proportion of people with a given illness or condition. The numerator is the number of new and pre-existing cases and the denominator is the size of the population. Point prevalence is the prevalence of an illness or condition at a particular instant in time. Prevalence is most useful for describing long-term or chronic illnesses, such as the long-term health consequences of ciguatera fish poisoning.

26.4.3 Measures of association

A measure of association quantifies the strength of the statistical association between the exposure and health outcome of interest. The calculated measure of association depends on the study design used (i.e. relative risk for a cohort study, odds ratio for a case-control study and prevalence ratio or prevalence odds ratio for cross-sectional studies). These measures of association are described in Dicker (1996) and are briefly discussed below.

The relative risk is the risk in the exposed group compared with the risk in the unexposed group (or background risk). The excess risk is expressed as a ratio. In our earlier example describing DSP among cruise ship passengers, 20 of the 53 people who claimed to have eaten steamed mussels became ill, whereas only two of the 69 people who did not eat any oysters became ill. The people who ate the oysters were 0.38/0.03 or 12.7 times more likely to become ill than those who did not eat the oysters.

In most case-control studies, the investigator does not know the true size of the exposed and unexposed populations, and no denominator is known by which an attack rate or a relative risk can be calculated. However, if the disease is rare, the relative risk can be approximated by the odds ratio which describes the chance of being exposed to a specific risk factor given disease status (ill or well) and is calculated from a two-by-two table (odds ratio = AD/BC) (Table 26.4). In the hypothetical example of DSP illnesses, the odds ratio was $(20 \times 67)/(33 \times 2)$ or 20.3.

Cross-sectional studies or surveys measure the prevalence (the number of existing cases) of a particular outcome or condition rather than the incidence (the number of new cases). The prevalence measures of association analogous to the risk ratio and odds ratio are the prevalence ratio and the odds ratio. For example, suppose that 20 of 100 people who reported an exposure, such as eating clams harvested from a specific beach during a specific time, became ill and that 20 of 380 people became ill who did not eat the clams. The prevalence ratio would be 0.20/0.05 = 4.0; that is, exposed people are 4.0 times more likely than unexposed people to have the illness. The prevalence odds ratio is $(20 \times 360)/(80 \times 20) = 4.5$. The odds of falling ill are 4.5 times higher for the exposed group than for the unexposed group.

TABLE 26.4 Layout and notation for a two-by-two table

	Ill	**Well**	**Total**	**Attack rate**
Exposed	A	B	A + B	A/(A + B)
Unexposed	C	D	C + D	C/(C + D)
Total	A + C	B + D	A + B + C + D	(A + C)/(A + B + C + D)

Source: Dicker (1996).

26.5 CLINICAL AND EPIDEMIOLOGICAL CHALLENGES

As mentioned throughout this chapter, a number of issues make the public health impact of exposure to marine algal toxins difficult to fully assess. In this section, some of these issues are summarized and examples are provided that might yield insight for future investigations.

26.5.1 Geographical distribution of marine phytoplankton species and related illnesses

The geographical distribution of specific marine phytoplankton species may vary because of aberrations in ocean currents, large-scale climate change or dispersion through anthropogenic activities such as transoceanic shipping. For example, the incidence of NSP in the USA is low because of routine plankton monitoring for *Karenia brevis* and for brevetoxins in seafood in areas where red tides typically occur. When shellfish become toxic, harvesting is closed. However, in 1987 an aberration in ocean currents swept an ongoing red tide from the Florida Gulf coast to the Atlantic coast of North Carolina (Morris *et al*., 1991), where routine monitoring was not conducted. Favourable conditions supported the development of an extensive red tide off the coast of North Carolina late in October. Because NSP had not been previously reported in North Carolina, the first official news release warning the public

not to eat shellfish did not occur until early November and 48 people became ill from eating contaminated oysters.

Another example was the PSP outbreak that occurred in Guatemala in July 1987 (Rodrigue *et al*., 1990). This toxin syndrome had not previously been identified in Guatemala and thus it was not properly diagnosed. In addition, there was no access to emergency facilities to provide respiratory life support. As a result, 26 (14%) of 187 cases, including 50% of ill children, died of respiratory arrest within hours of symptom onset.

The geographical distribution of human illness associated with exposure to toxic marine algae is increasing in scope. International travel, seafood importation and tourism increase people's exposure to exotic seafood potentially contaminated by marine algal toxins (Todd, 1994). Human activities and periodic changes in global climate and ocean currents can affect the distribution of marine phytoplankton, and thus affect which populations are at risk from these diseases.

26.5.2 Clinical diagnoses

The differential diagnosis for acute illness from these toxins includes allergic reaction to seafood, food poisoning from other sources, organophosphate or carbamate pesticide poisoning or scombroid fish poisoning. For example, the gastrointestinal symptoms from anticholinesterase pesticide poisoning (nausea, vomiting) could be confused with several of these illnesses, including PSP (see Kao, 1993). However, symptoms of pesticide poisoning, but not of PSP, would be relieved following the administration of atropine. Other symptoms associated with anticholinesterase pesticide poisoning, such as excessive salivation, lacrimation, bronchial secretions and pupil constriction would differentiate it from PSP.

Another intoxication that may present with symptoms similar to PSP is poisoning associated with the puffer fish (*fugu*). Although tetrodotoxin is not closely related to PSP toxins in structure, the neurological manifestations of intoxication resemble those of PSP (Kao, 1993). Early appearance of nausea and vomiting may occur more often with *fugu* and hypotension is almost always associated with this toxin but not with PSP. Lastly, botulism caused by toxins produced by *Clostridium botulinum* also causes flaccid paralysis, but it has a much longer incubation period (days).

In addition to acute symptoms, several of these diseases have chronic symptoms. For example, ASP is associated with apparently permanent memory loss (Perl *et al*., 1990). People who do not receive treatment for CFP within 72 hours complain of paresthesias and other neurological symptoms for weeks to months (Blythe *et al*., 1994). The differential diagnosis for chronic illness from these toxins includes chronic pesticide poisoning, encephalitis (especially for ASP) and multiple sclerosis (especially for CFP). In addition, chronic HAB-associated diseases can be misdiagnosed as psychiatric disorders or other chronic diseases (Pearn, 1994).

26.5.3 Toxin characteristics

Changes in the specific symptoms reported by individuals with these diseases can also vary geographically. For example, in Polynesia, the neurological symptoms of CFP predominate and appear before the gastrointestinal symptoms. By contrast, Caribbean residents with CFP typically present acute gastrointestinal symptoms that may be associated with cardiovascular symptoms, with a more gradual onset of the

neurological symptoms (Bagnis *et al*., 1979; Morris *et al*., 1982*a*, 1982*b*; Lawrence *et al*., 1980; Blythe *et al*., 1994). On Réunion in the south-west Indian Ocean, 16% of people with the disease report hallucinatory poisoning (lack of co-ordination, loss of equilibrium, hallucinations, mental depression and nightmares) in addition to the symptoms reported by victims in the Pacific and Caribbean (Quod and Turquet, 1996). These differences in the course and symptoms of the illness are probably related to different toxin mixtures produced by different strains of *G. toxicus* (Baden *et al*., 1995).

Although CFP has some distinctive clinical features, the reported symptoms vary among individuals, suggesting that there are several closely related but distinct toxins (Glaziou and Legrand, 1994) making it difficult to diagnose. Escalonta de Motta *et al*. (1986) conducted a study to examine the occurrence and clinical manifestations of the disease in Puerto Rico. They found that, of people who reported gastrointestinal symptoms (nausea, vomiting, diarrhoea or intestinal pain) and at least two neurological alterations (malaise, pain, paresthesias, temperature inversion, metallic taste or pruritus), approximately 83% reported at least one gastrointestinal symptom, 65% reported malaise and 54% reported finger paresthesias. However, only 48% reported temperature-sensation reversal. Similarly, in other studies of Caribbean populations, Morris *et al*. (1982*b*) found that over 80% of affected people reported gastrointestinal symptoms and 30–40% reported paresthesias; fewer than 40% of these individuals reported temperature-sensation reversal. Thus the symptom that would be the most useful in diagnosing CFP, the unique temperature-sensation reversal, occurs in fewer than half of the poisoning victims.

26.5.4 Verifying exposure

The symptoms of the diseases caused by marine algal toxins are primarily non-specific, therefore a biological marker would be useful to indicate exposure or to identify a unique effect in humans. Unfortunately, human biological markers of exposure to these toxins and their subsequent biological effects are experimental or non-existent (Fleming *et al*., 1995).

In the past, identifying the specific food source or transvector (if available), was part of the clinical diagnosis. This is reflected in the names of these diseases (i.e. ASP, DSP, NSP and PSP from eating contaminated shellfish and CFP from eating contaminated fish). However, new research has demonstrated that these toxins can accumulate in other transvectors, including animals and the environment. For example, domoic acid, which is associated primarily with shellfish and the occurrence of ASP in humans, also causes disease in marine mammals, such as sea lions, that eat contaminated fish as well as shellfish (Scholin *et al*., 2000).

The levels of toxin(s) and the potency of the toxin(s) in the transvector can be measured, but there is rarely a sample available (see Chapters 8–13 for methods of detecting algal toxins). As part of confirming the clinical diagnosis of illnesses associated with HABs, algal cell counts and toxin levels could be obtained from ambient water samples.

26.5.5 Emerging issues

Although some of the diseases caused by marine microalgae have been known for centuries, few epidemiological studies of the etiology, long-term health consequences or prevention of these diseases have been conducted. In addition, because

the geographical distribution of these marine organisms is changing over time and because the toxins they produce are typically families of similar chemicals rather than one specific compound, investigators are likely to encounter future outbreaks associated with organisms and/or toxins that have not been reported.

A recent example is human exposure to the, as yet undescribed, toxins produced by *Pfiesteria piscicida*, a dinoflagellate that has been found in estuaries along the Atlantic and Gulf Coasts of the USA. There have been reports of human illness associated with occupational exposures to concentrated laboratory cultures of *P. piscicida* (Glasgow *et al*., 1995). The presence of the organism has been associated with the production of ulcerative lesions as well as massive fill kills in rivers and estuaries (Burkholder *et al*., 1992). In addition to the published reports, there is anecdotal evidence that environmental exposure to this organism may affect human health. These reports generated concern that environmental exposure to *P. piscicida* and/or toxins produced by the organism could cause adverse human health effects. However, efforts to identify a toxin elaborated by *P. piscicida* have not yet been successful and, without something to measure, confirming an environmental exposure or associated human illness is not possible (CDC, 2000).

26.6 Disease prevention and public health intervention

As discussed above, epidemiological field investigations are typically conducted in response to an acute problem. However, these investigations may identify more long-term problems that warrant further investigation or the institution of control measures to protect public health and prevent new cases. For example, for the diseases caused by exposure to marine algal toxins, an outbreak is typically a disease cluster because many members of a village or several diners at a restaurant eat portions of the same seafood, or a group of people may have been on the beach during a red tide. An investigation of even a single case of one of these illnesses will probably identify additional cases and provide the opportunity for appropriate medical follow-up. An investigation of the contaminated transvector may reveal, for example, that shellfish harvested in a particular estuary are toxic. In this case, health officials can prevent new cases by disposing of the contaminated food or legally closing shellfish harvesting until the animals are no longer toxic, thus preventing new cases.

Another public health intervention would be to prevent exposure. For example, in Miami, Florida, barracuda have been found to be so frequently contaminated with ciguatera toxins that city ordinances forbid the sale of this fish (Lawrence *et al*., 1980). Shellfish monitoring programmes (see Chapters 23 and 24) allow environmental health authorities to identify when shellfish begin accumulating toxins and to close the area to harvesting. However, preventing human exposure in this way may not always be possible. For example, in areas where CFP is endemic and where people rely on subsistence fishing, local health agencies may consider the low death rates from ciguatera relatively unimportant when compared with those from other important public health problems, such as respiratory and other gastrointestinal illnesses (Dalzell, 1994). Public health activities may be limited to publishing the names of the potentially toxic species. However, even in these areas, an effective approach may be to increase the supply of non-toxic fish by targeting species that

live beyond the reef and are thus less likely to be toxic. The South Pacific Commission has successfully introduced the different technologies needed to catch deep-slope fishes in several countries (Dalzell, 1994).

Programmes to educate and inform the public, physicians and other health-care providers about the diseases associated with marine algal toxins are needed to improve diagnosis and reporting. Presentations at local or regional epidemiology or medical society meetings may be useful for making physicians aware of the signs and symptoms. Easily accessible information (pamphlets, public service announcements, websites) could educate the public about the health risks associated with consuming seafood. Early diagnosis may prevent additional cases and allow appropriate treatments to be provided in a timely manner. Furthermore, recognizing that a patient is ill with a disease caused by marine algal toxins would allow a physician to alert public health officials so that they can initiate prevention and control activities.

26.7 Conclusion

Under-diagnosis and under-reporting (even in endemic areas) pose problems in determining the true worldwide incidence of human illness associated with marine algal toxins. In addition, almost no research has been conducted on the possible chronic health effects of exposure to these marine biotoxins, either after an acute exposure or after long-term low-level exposure. Surveillance for human diseases associated with marine microalgae would be invaluable in calculating their public health impact. However, until they are recognized and correctly diagnosed in areas where they are rare, and more efficiently documented in areas where they are endemic, their full public health impact will continue to be underestimated.

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Part IV

Appendices

Appendix A

A world list of algal culture collections

Prepared by R. A. Andersen

The purpose of this appendix is to provide a list of algal culture collections, especially those that maintain harmful algae. The collections are organized alphabetically by country and by the name of the culture collection within each country. The list is restricted to those collections that can be contacted by electronic methods, i.e. e-mail and Internet websites. Electronic addresses change more frequently than postal addresses and, although every effort has been made to insure that they are correct, changes will occur. In some cases, the website is the host institution of the collection and the collection itself has no website. The term 'curator' is applied broadly and the tasks of the curator vary markedly from collection to collection.

Each collection, public or private, may have restrictions regarding the distribution and shipping of strains, especially toxic strains. I have not listed these restrictions and users should contact collections directly to determine if restrictions apply. Most public collections sell cultures. When ordering cultures, first try the culture collection within your own country, if one exists. The transit time for a culture is almost always less within a country and there are usually no customs requirements when cultures are shipped within a country (Hawaii, however, imposes state import restrictions for cultures shipped within the USA). If cultures are shipped between countries, customs documentation is necessary. Customs officials are not always sympathetic when improper customs documentation is used and delays at customs offices often result in the death of the organisms. In general, whether within or between countries, it is prudent to use 'overnight' courier services (e.g. Federal Express, DHL, TNT International). These couriers provide a rapid service with tracking systems to locate quickly delayed shipments. Lastly, good communications between the user and the collection are recommended. Curators of collections in one country may not be aware of holidays in other countries and are unlikely to know when the user is present or absent from their institution.

Many dinoflagellate and raphidophyte strains are difficult to transport and in some cases successful shipment is impossible. Dinoflagellates have a high respiration rate relative to their stored food and the cells 'starve' when kept in the dark for more than one or two days. Dinoflagellates are also sensitive to dramatic photoperiod changes. Raphidophytes, especially *Chattonella* spp., are extremely difficult to ship successfully, although limited success can be achieved when the cell density is low.

The proper culture medium and the appropriate culture conditions (e.g. incubator temperature and lighting) should be prepared in advance. For culturing details, see Chapter 3. On arrival of the cultures, users should immediately examine them for viability, remembering that, as a result of shipment, normally swimming organisms

may be settled to the bottom. When preparing a subculture from the stock, users should maintain a portion of the original stock culture in its original container, i.e. do not pour all the stock into your culture medium.

It is assumed that readers will understand that strict laboratory precautions should be employed when handling toxic phytoplankton and that they should be very careful when disposing of cultures of toxic phytoplankton. It is often illegal to dispose of phytoplankton (toxic or otherwise) into natural waters. In cases where such releases are not illegal, it is generally considered undesirable to release phytoplankton into the local environment.

ARGENTINA

Coleccion de Cultivo de Algas de los Laboratorios de Ficologia

Departamento de Ciencias Biologicas, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, 1428 Buenos Aires, Argentina
Curator: Juarez, A. B.
E-mail: abjuarez@bg.fcen.uba.ar
Tel.: +54 11-4576-3349
Fax: +54 11-4576-3384
Website: none

AUSTRALIA

Australian Collection of Antarctic Microorganisms (ACAM)

CRC for the Antarctic and Southern Ocean Environment and the School of Agricultural Science, University of Tasmania, GPO Box 252-54, Hobart, Tasmania 7001, Australia
Curator: Nichols, C. M.
E-mail: acam@agsci.utas.edu.au
Tel.: +61 3-6226-2776
Fax: +61 3-6226-2642
Website: http://www.antcrc.utas.edu.au/antcrc/micropro/acaminfo.html

Australian Collection of Marine Microorganisms (ACMM)

Australian Institute of Marine Science, PMB 3, Townsville MC, Cape Ferguson, Queensland 4810, Australia
Curator: Llewellyn, L. E.
E-mail: l.llewellyn@aims.gov.au
Tel.: +61 7-4753-4449
Fax: +61 7-4753-4285
Website: http://www.aims.gov.au/

Australian Collection of Microorganisms (ACM)

Department of Microbiology and Parasitology, University of Queensland, Brisbane, Queensland 4072, Australia

Director: Sly, L. I.
E-mail: sly@biosci.uq.edu.au
Tel.: +61 7-3365-2396
Fax: +61 7-3365-1566
Website: http://www.biosci.uq.edu.au/micro/culture/culture.htm

Australian Water Quality Centre Culture Collection (AWQC)

Australian Water Quality Centre, Private Mail Bag 3, Salisbury, South Australia 5108, Australia
Curator: Baker, P.
E-mail: peter.baker@sawater.sa.gov.au
Tel.: +61 8-8259-0238
Fax: +61 8-8259-0228
Website: http://www.awqc.com.au

CSIRO Collection of Living Microalgae

CSIRO Marine Research, Castray Esplanade, GPO Box 1538, Hobart, Tasmania 7001, Australia
Director: Blackburn, S.
Curator: Leroi, J.-M.
E-mail: microalgae@marine.csiro.au
Tel.: +61 3-6232-5306
Fax: +61 3-6232-5471
Website: http://www.marine.csiro.au/microalgae/collection.html

Harmful Algae Culture Collection (UTAS)

Aquatic Botany, School of Plant Science, University of Tasmania, GPO Box 252-55, Hobart, Tasmania 7001, Australia
Curator: Marshall, J.
E-mail: Judi.Marshall@utas.edu.au
Tel.: +61 3-6226-2623
Fax: +61 3-6226-2698
Website: none

AUSTRIA

Culture Collection of Algae (ASIB)

University of Innsbruck, Institut für Botanik, Sternwartestrasse 15, 6020 Innsbruck, Austria
Curator: Gaertner, G.
E-mail: Georg.Gaertner@uibk.ac.at
Tel.: +512 507-5939
Fax: +512 293439
Institutional website: http://info.uibk.ac.at/

BELGIUM

Microalgal Culture Collection

University of Liege, Genetics of Microorganisms, Department of Botany, 4000 Liege, Belgium
Curator: Matagne, R.
E-mail: rf.matagne@ulg.ac.be
Tel.: +324 3663-820/27
Fax: +324 3663-840
Website: none

BRAZIL

Faculdade de Ciências e Letras de Assis-Universidade Estadual Paulista (FCLA-UNESP)

Av. Dom Antonio 2100, Dep. Ciências Biológicas, Assis, SP 19800-000, Brazil
Curator: do Carmo Bittencourt-Oliveira, M.
E-mail: mbitt@assis.unesp.br
Tel.: +55 18-322-2933 r. 265
Fax: +55 18-322-2933 r. 320
Website: http://www.assis.unesp.br/~mbitt

CANADA

Canadian Centre for the Culture of Microorganisms (NEPCC)

Department of Botany, University of British Columbia, 6270 University Boulevard, Vancouver, BC V6T 1Z4, Canada
Director: Borden, C.-A.
Curator: Dinh, D.
E-mail: cccm@interchange.ubc.ca
Tel.: +1 604-822-4825
Fax: +1 604-822-6089
Website: http://www.ocgy.ubc.ca/projects/nepcc/

University of Toronto Culture Collection of Algae and Cyanobacteria (UTCC)

Department of Botany, University of Toronto, Toronto, Ontario M5S 3B2, Canada
Curator: Acreman, J.
E-mail: jacreman@botany.utoronto.ca
Tel.: +1 416-978-3641
Fax: +1 416-978-5878
Website: http://www.botany.utoronto.ca/utcc/

China

Collection of Marine Biological Germplasm

Institute of Oceanology, Chinese Academy of Sciences,
7th Nanhai Road, Qingdao, Shandong 266071, People's Republic of China
Director: Hu, Xiao-Yan
E-mail: xyhu@ms.qdio.ac.cn
Tel.: +86 532-287-9062 Ext. 4311
Fax: +86 532-287-0882
Website: http://www1.im.ac.cn/typecc/index.html/

University of Hong Kong Algal Culture Collection (HKUACC)

Department of Ecology & Biodiversity, University of Hong Kong, Pokfulam Road, Hong Kong, People's Republic of China
Curator: Lu, S.
E-mail: shlu@hkusua.hku.hk
Tel.: +852 2299-0683
Fax: +852 2517-6082
Website: none

Czech Republic

Culture Collection of Autotrophic Organisms (CCALA)

Academy of Sciences of Czech Republic, Institute of Botany, Culture Collection of Autrophic Organisms, Dukelska 135, CZ-379 82 Trebon, Czech Republic
Curator: Lukavsky, J.
E-mail: lukavsky@butbn.cas.cz
Tel.: +420 333-721156
Fax: +420 333-721136
Website: http://www.butbn.cas.cz/ccala/ccala.htm

Denmark

Scandinavian Culture Center for Algae and Protozoa (SCCAP)

University of Copenhagen, Botanical Institute, Øster Farimagsgade 2D, DK 1353 Copenhagen K, Denmark
Curator: Larsen, N.
E-mail: SCCAP@BOT.KU.DK
Tel.: +45 35322303/2292
Fax: +45 35-322321
Website: http://www.sccap.bot.ku.dk/

FRANCE

Algothec (ALGOBANK)

Laboratoire de Biologie et Biotechnologies Marines, Université de Caen, Basse-Normandie, BP 5186, 14032 Caen, France
Curator: Veron, B.
E-mail: veron@ibba.unicaen.fr
Tel.: +33 2-31-56-58-37
Fax: +33 2-31-56-53-46
Website: http://www.unicaen.fr/unicaen/ufr/ibba/lbbm/algobank/sommaire.html

Algothèque du Laboratoire de Cryptogamie

Museum National d'Histoire Naturelle, 12 rue Buffon, 75005 Paris, France
Curator: Couté, A.
E-mail: acoute@mnhn.fr
Tel.: +33 1-40-79-31-96
Fax: +33 1-40-79-35-94
Website: http://www.mnhn.fr

Pasteur Culture Collection of Cyanobacteria (PCC)

Unité des Cyanobactéries, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France
Curator: Rippka, R.
E-mail: rrippka@pasteur.fr; mherdman@pasteur.fr
Tel.: +33 1-45-68-84-16
Fax: +33 1-40-61-30-42
Website: http://www.pasteur.fr/recherche/banques/PCC/

Roscoff Culture Collection (RCC)

Station Biologique CNRS/INSU/UPMC, BP 74, 29682 Roscoff, France
Curator: Vaulot, D.; Le Gall, F.
E-mail: vaulot@sb-roscoff.fr
Tel.: +33 2-98-29-23-34
Fax: +33 2-98-29-23-24
Website: http://www.sb-roscoff.fr/Phyto/collect.html

GERMANY

Culture Collection of Cryophilic Algae (CCCryo)

Institute of Biology — Membrane Physiology, Humboldt-University Berlin, Invalidenstrasse 42, 10115 Berlin, Germany
Curator: Leya, T.
E-mail: thomas.leya@rz.hu-berlin.de
Tel.: +49 30-2093-8338
Fax: +49 30-2093-8359
Website: http://www.ibmt.fhg.de/ibmt3ambtbiotechnolextremophilen_index.html

Phytoplankton Kulturensammlung Biologische Anstalt Helgoland (BAHME)

Wattenmeerstation Sylt des Alfred-Wegener-Instituts für Polar- und Meeresforschung
Hafenstrasse 43, D-25992 List/Sylt, Germany
Curator: Elbraechter, M.
E-mail: melbraechter@awi-bremerhaven.de
Tel.: +49 4651-870408/4651-956150
Fax: +49 4651-870408/5651-956200
Website: none

Sammlung von Algenkulturen (SAG)

Albrecht-von-Haller-Institut für Pflanzenwissenschaften, Abteilung Experimentelle Phykologie und Sammlung von Algenkulturen, Universitaet Goettingen, Untere Karspuele 2, 37073 Goettingen, Germany
Director: Friedl, T.
E-mail: tfriedl@gwdg.de
Tel.: +49 551-397868/397870
Fax: +49 551-397871/397823
Website: http://www.gwdg.de/~epsag/phykologia/sag/sag.htm

IRELAND

Seaweed Culture Collection (GALW)

Martin Ryan Marine Science Institute, National University of Ireland, Galway, Ireland
Curator: Guiry, M.
E-mail: mike.guiry@nuigalway.ie
Tel.: +359 91-750410
Fax: +359 91-525005
Website: www.seaweed.ie

ITALY

Cyanobacteria Culture Collection (CNR)

CNR, Centro di Studio dei Microorganismi Autotrofi, P. le Delle Cascine 27, 50144 Firenze, Italy
Curator: Tredici, M.
E-mail: tomaselli@csma.fi.cnr.it
Tel.: +39 55-328806
Fax: +39 55-330431
Website: none

JAPAN

Culture Collection (IAM)

Center for Bioinformatics, Institute of Molecular and Cellular Biosciences, University of Tokyo 1-1, Yayoi 1-chome, Bunkyo-ku, Tokyo 113-0032, Japan
Curator: Yokota, A.
E-mail: iamcc@iam.u-tokyo.ac.jp
Tel.: +81 3-5841-7827
Fax: +81 3-5841-8490
Website: http://www.iam.u-tokyo.ac.jp/misyst/ColleBOX/IAMcollection.html

Marine Biotechnology Institute Culture Collection (MBIC)

Marine Biotechnology Institute, Kamaishi Laboratory, 3-75-1 Heita, Kamaishi, Iwate 026-0001, Japan
Curator: Suda, S.
E-mail: shoichiro.suda@kamaishi.mbio.co.jp
Tel.: +81 193-26-6538
Fax: +81 193-26-6584
Website: http://www.mbio.co.jp/mbic

Microbial Culture Collection (NIES)

National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan
Director: Watanabe, M. M.
Curator: Kasai, F.
E-mail: mcc@nies.go.jp
Tel.: +81 298-50-2556
Fax: +81 298-50-2587
Website: http://www.nies.go.jp/labo/mcc/home.htm

MALAYSIA

University of Malaya Algae Culture Collection (UMACC)

Institute of Biological Sciences, University of Malaya, 50603 Kuala Lumpur, Malaysia
Curator: Phang Siew Moi
E-mail: phang@biology.um.edu.my, h1phangs@umcsd.um.edu.my
Tel.: +603 7967-4610
Fax: +603 7967-4606
Website: none

NETHERLANDS

Culture Collection (NICMM)

Box 8039, 4330 EA Middleburg, the Netherlands
Curator: Peperzak, L.
E-mail: L.Peperzak@rikz.rws.minvenw.nl
Tel.: +31 118-672332
Fax: +31 118-651046
Website: none

NEW ZEALAND

Cawthron Culture Collection of Microalgae (CAW)

Cawthron Institute, 98 Halifax Street East, Private Bag 2, Nelson, New Zealand
Curator: Ponikla, K.
E-mail: Krystyna@cawthron.org.nz/Lesley@cawthron.org.nz
Tel.: +64 3-548-2319
Fax: +64 3-546-9464
Website: none

Marine Phytoplankton Culture Collection (NIWA)

National Institute of Water and Atmospheric Research Ltd., PO Box 14-901, Kilbirnie, Wellington, New Zealand
Curator: Chang, F. Hoe
E-mail: h.chang@niwa.cri.nz
Tel.: +64 4-386-0300
Fax: +64 4-386-2153
Website: http://www.niwa.cri.nz/pgsf/MarineBiodiversity/

NORWAY

NIVA Culture Collection (NIVA)

Norwegian Institute for Water Research, PO Box 173, Kjelsås, N-0411 Oslo, Norway
Curator: Skulberg, O. M.
E-mail: olav.skulberg@niva.no
Tel.: +47 22-185100
Fax: +47 22-35200
Website: http://www.niva.no

PHILIPPINES

University of the Philippines Culture Collection (MSIUP)

Marine Science Institute, University of the Philippines, Quezon City 1101, the Philippines

Curator: Azanza, R. V.
E-mail: rhod@upmsi.ph
Tel.: +632 921-5967
Fax: +632 921-5967
Website: http://www.upmsi.ph

PORTUGAL

Algoteca de Coimbra Culture Collection (ACOI)

Departamento de Botânica, Universidade de Coimbra, 3000 Coimbra, Portugal
Curator: Santos, M. F.
E-mail: mfsantos@ci.uc.pt
Tel.: +351 23-982-28-97
Fax: +351 23-982-07-80
Website: http://www.uc.pt/botanica/ACOI.htm

Marine Cyanobacteria Culture Collection (CIMARCYA)

Centre for Marine and Environmental Research, Porto University, R. Campo Alegre 823, 4150-180 Portugal
Curator: Vasconcelos, V.
Tel.: +351 22-606-04-21
Fax: +351 22-606-04-23
Website: none

Microalgae Culture Collection-Biotechnology Plant Laboratory

Escola Superior de Biotecnologia — Universidade Católica do Porto,
R. Dr Antonio Bernardino de Almeida, 4200-072 Portugal
Curator: Raposo, M. F. de Jesus
E-mail: fraposo@esb.ucp.pt
Tel.: +351 22-558-00-01 Ext. 1338
Fax: +351 22-509-03-51
Website: none

RUSSIAN FEDERATION

Collection of Algal Cultures of Leningrad University (CALU)

Biological Institute of St Petersburg State University, Oranienbaumskoye sch. 2, 198504 St Petersburg, Stariy Peterhof, Russian Federation
Director: Gromov, B. V.
Curator: Titova, N. N.
E-mail: Boris.Gromov@paloma.spbu.ru
Tel.: +812 427-97-40
Fax: +812 427-73-10
Website: none

Collection of Microalgae (IPPAS)

Russian Academy of Sciences, Inst. of Plant Physiology, Botanicheskaya 35, Moscow, Russian Federation
Curator: Kuptsova, E.
E-mail: kuptsova@ippras.ru
Tel.: +795 977-94-27
Fax: +795 977-80-18
Website: http://www.ippras.ru/ippasmain_e.htm

South Africa

University of the Witwatersrand Culture Collection (WITS)

School of Animal, Plant & Environmental Sciences, University of the Witwatersrand, Private Bag 3, Wits 2050, South Africa.
Curator: Pienaar, R.
E-mail: Richard@gecko.biol.wits.ac.za
Tel.: +27 11-717-6423
Fax: +27 11-403-1429
Website: none

Spain

Colección de cultivos de microalgas nocivas del IEO en Vigo (CCVIEO)

Instituto Español de Oceanografía, Subida á Radiofaro, 50, 36390 Vigo, Spain
Director/*Curator*: Fraga, S.
E-mail: santiago.fraga@vi.ieo.es
Tel.: +34 986-492111
Fax: +34 986-492351
Website: http://www.ieo.es/

Marine Microalgae Culture Collection (ICMAN-CSIC)

Instituto Ciencias Marinas de Andalucia (Consejo Superior de Investigaciones Científicas) Campus Universitario, Rio San Pedro S/N, 11501 Puerto Real, Cadiz, Spain
Curator: Lubian, L. M.
E-mail: Luis.Lubian@icman.csic.es
Tel.: +34 56-832612
Fax: +34 56-834701
Website: http://www.icman.csic.es/

Sweden

Kalmar Algae Collection (KAC)

Department of Marine Science, University of Kalmar, S-391 82 Kalmar, Sweden

Curator: Lindquist, C. E.
E-mail: christina.esplund@hik.se/ch_esplund@hotmail.com
Tel.: +46 480-447329
Fax: +46 480-447305
Website: http://www.bom.hik.se/~nesch/KACkatalog.html

THAILAND

BIOTEC Culture Collection (BIOTEC-Thailand)

National Center for Genetic Engineering and Biotechnology, 73/1 Rama 6 Road, Patumwan, Bangkok 10400, Thailand
Curator: Potacharoen, Wanchern
E-mail: wanchern@biotec.or.th
Tel.: +66 2-6448150 Ext. 543
Fax: +66 2-6448107
Website: none

TISTR Culture Collection (TISTR)

Thailand Institute of Scientific and Technological Research, 196 Phahonyothin Road, Chatuchak, Bangkok 10900, Thailand
Curator: Arunpairojana, Vullapa
E-mail: mircen@tistr.or.th, tistr@mozart.inet.co.th
Tel.: +66 5790160/5795515
Fax: +66 5614771/5799542
Website: none

UNITED KINGDOM OF GREAT BRITAIN AND NORTHERN IRELAND

Culture Collection of Algae and Protozoa (CCAP, Freshwater)

United Kingdom Federation of Culture Collections (UKFCC), Institute of Freshwater Ecology, The Windermere Laboratory, Far Sawrey, Ambleside, Cumbria LA22 0LP, UK
Director: Day, J. J.
E-mail: ccap@ife.ac.uk
Tel.: +44 01539-442-468
Fax: +44 01539-446-914
Website: http://www.ife.ac.uk/ccap/straindata.html

Culture Collection of Algae and Protozoa (CCAP, Marine)

Scottish Association for Marine Science, Dunstaffnage Marine Laboratory, Oban, Argyll PA34 4AD, Scotland, UK
Director: Bolch, C.
Curator: Campbell, C.
E-mail: ccapn@dml.ac.uk, cjsb@dml.ac.uk

Tel.: +44 01631-559-268
Fax: +44 01631-559-001
Website: www.ife.ac.uk/ccap

Edinburgh Collection of Algal Cultures (ECAC)

Royal Botanic Garden, Edinburgh EH3 5LR, Scotland, UK
Curator: Mann, D. G.
E-mail: d.mann@rbge.org.uk
Tel.: +44 0131-248-2875
Fax: +44 0131-248-2901
Website: none

Plymouth Culture Collection (PLY)

Plymouth Marine Laboratory, Citadel Hill, Plymouth PL1 2PB, UK
*Curator*s: Pipe, R; Jutson, M.
E-mail: rkpi@mail.pml.ac.uk, mgsj@mail.pml.ac.uk
Tel.: +44 01752-633-216
Fax: +44 01752-633-102
Website: none

University of Dundee Culture Collection (UDUN)

University of Dundee, Department of Biological Sciences, Dundee, Scotland, UK
Curator: Codd, G. A.
E-mail: g.a.codd@dundee.ac.uk
Tel.: +44 01382-344-272
Fax: +44 01382-344-275
Website: http://www.dundee.ac.uk/bioscience/codd.htm

University of Westminster Algal Collection (UW)

University of Westminster, 115 New Cavendish Street, London W1M 5JS, UK
Curator: Lewis, J.
E-mail: lewisjm@westminster.ac.uk
Tel.: +44 020-7911-5000 Ext. 3800
Fax: +44 020-7911-5087
Website: none

United States of America

Loras College Freshwater Diatom Culture Collection (FDCC)

Department of Biology, Loras College Dubuque, IA 52004-0178, USA
Curator: Czarnecki, D.B.
E-mail: czdiatom@loras.edu
Tel.: +1 563-588-7231
Fax: +1 563-588-7964
Website: http://www.bgsu.edu/Departments/biology/algae/html/DiatomCulture.html

Northwest Fisheries Science Center Culture Collection of Algae

NMFS/NWFSC/BIOTOX 2725 Montlake Blvd E. Seattle, WA 98112, USA
Curator: Trainer, V. L.
E-mail: vera.l.trainer@noaa.gov
Tel.: +1 206-860-6788
Fax: +1 206-860-3335
Website: http://www.nwfsc.noaa.gov/hab

Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP)

Bigelow Laboratory for Ocean Sciences, 180 McKown Point Road, West Boothbay Harbor, ME 04575, USA
Director: Andersen, R. A.
Curator: Sexton, J. P.
E-mail: ccmp@bigelow.org, randersen@bigelow.org
Tel.: +1 207-633-9630/9600
Fax: +1 207-633-9715/9641
Website: http://ccmp.bigelow.org

University of Rhode Island Culture Collection (URI)

Graduate School of Oceanography, Univ. of Rhode Island, RI 02882-1197, USA
Curator: Hargraves, P.
E-mail: pharg@gso.uri.edu
Tel.: +1 401-874-6241
Fax: +1 401-872-6240
Website: none

University of Texas Culture Collection of Algae (UTEX)

MCD-Biology, University of Texas, Austin, TX 78712, USA
Director: Brand, J.
E-mail: utalgae@uts.cc.utexas.edu; jbrand@utxsvs.cc.utexas.edu
Tel.: +1 512-471-4019
Fax: +1 512-471-0354
Website: http://www.bio.utexas.edu/research/utex/

Woods Hole Culture Collection (WHOI)

Woods Hole Oceanographic Inst., Biology Dep., Redfield 3-32, Woods Hole, MA 02543, USA
Curator: Kulis, D.
E-mail: dkulis@whoi.edu
Tel.: +1 508-289-2859
Fax: +1 508-457-2027
Website: none

Appendix B

Agencies and addresses: international and regional organizations with programmes or activities on harmful microalgae

Prepared by H. O. Enevoldsen

This appendix aims to facilitate communication between scientists, managers and the various international or regional organizations that are running programmes on harmful microalgae. As the activities and scope of science and management programmes are continually evolving, only very basic information is given. For detailed up-to-date information, the relevant organization should be contacted directly. Only organizations whose explicit priority is harmful microalgae and with continual activities in the field are included. In addition to these, several organizations or international development agencies run short- or long-term projects related to HAB on a case-to-case basis. A supplement to this appendix is the IOC database HABDIR, an International Directory of Experts in Harmful Algae and their Effects on Fisheries and Public Health, available on-line at the IOC website (http://ioc.unesco.org/).

INTERGOVERNMENTAL ORGANIZATIONS

Intergovernmental Oceanographic Commission (IOC) of UNESCO

Background

The IOC was set up by UNESCO in 1960 to develop, recommend and co-ordinate international programmes for the scientific investigation of the oceans and to provide related ocean services to Member States (currently 126). Within the United Nations, the IOC alone has the responsibility for basic oceanographic research. It is also the UN focal agency for the implementation of Agenda 21, Chapter 17, on the oceans. A functionally autonomous body within UNESCO, the IOC collaborates with other organizations within and outside the UN system.

IOC Harmful Algal Bloom Programme

IOC Member States initiated a Harmful Algal Bloom Programme in 1992. Through a number of international workshops, a programme plan was prepared to cover educational, scientific and operational aspects of harmful algae. Activities are continuously developed, and since 1992 implemented jointly by IOC Member States, the IOC Secretariat, and co-sponsoring organizations through the IOC Intergovernmental Panel on Harmful Algal Blooms (IPHAB), which was established to provide an

international framework for HAB-related activities implemented by the broad international community.

Goal

'To foster the effective management of, and scientific research on, harmful algal blooms in order to understand their causes, predict their occurrences, and mitigate their effects.'

Activities

Educational activities include training courses on taxonomy, toxin determination and management of harmful algae, individual study grants, publication of a newsletter on toxic algae and algal blooms (*Harmful Algae News*), manuals, guides and directories on various topics related to harmful algae.

Scientific activities includes the international IOC-SCOR research programme on the global ecology and oceanography of harmful algal blooms, GEOHAB; an ICES-IOC Working Group on Harmful Algal Bloom Dynamics, Regional Science Planning Workshops, and related pilot projects and workshops. To facilitate implementation of training and capacity building activities, IOC Science and Communication Centres on HAB are established at the University of Copenhagen, Denmark, and at the Spanish Institute of Oceanography in Vigo, Spain. Operational activities include initiatives directed towards improved resource protection, monitoring, public health and seafood safety. At the regional level, the IOC has HAB working groups in the Western Pacific (WESTPAC/HAB), South America (FANSA) and the Caribbean (ANCA).

Databases, publications, announcements of training courses, as well as general information is available from the IOC HAB Internet home page at http://ioc.unesco.org/hab/.

Liaisons

Joint activities primarily with SCOR, ICES, IMO, regional organizations and national research institutions.

Focal points

Intergovernmental Oceanographic Commission, Harmful Algal Bloom Programme Office, UNESCO, 1, rue Miollis, 75732 Paris Cedex 15, France
Fax: +33 1-40-56-93-16
E-mail: hab.ioc@unesco.org
Website: http://ioc.unesco.org/iocweb/

IOC/WESTPAC, c/o National Research Council Thailand, 196 Phaholyothin Road, Chatujak, Bangkok 10900, Thailand
Tel.: +66 2-561-5118/9
Fax: +66 2-561-5119
E-mail: westpac@samart.co.th

IOC Regional Office for the Caribbean – IOCARIBE, Apartado Aereo 1108, Casa del Marques de Valdehoyos, Cartagena, Colombia
Tel.: +57 5-364-6399
Fax: +57 5-364-6399
E-mail: C.Toro@unesco.org

IOC Science and Communication Centre on Harmful Algae, University of Copenhagen, Øster Farimagsgade 2D, DK-1353 Copenhagen K, Denmark
Tel.: +45 33-134446
Fax: +45 33-134447
E-mail: hab@bot.ku.dk

IOC-IEO Science and Communication Centre on Harmful Algae, Instituto Español de Oceanografia, Centro Oceanográfico de Vigo, Cabo Estay-Canido, Apdo 1552, E-36280 Vigo, Spain
Tel: +34 986-492111
Fax: +34 986-492003
E-mail: vigohab@vi.ieo.es

IOC website: http://ioc.unesco.org/hab/

International Atomic Energy Agency (IAEA) of the United Nations

Background and activities

The IAEA is an independent, intergovernmental, science and technology-based organization, in the United Nations family, that serves as the global focal point for nuclear co-operation. The IAEA assists its Member States, in the context of social and economic goals, in planning for and using nuclear science and technology for various peaceful purposes.

The IAEA has technical co-operation projects related to HABs currently active or planned at the national, regional and interregional levels. The initial major focus of these activities is technology transfer of a competitive receptor-binding assay for neurotoxins, such as saxitoxin, that uses radiometric detection. The agency's Marine Environment Laboratory in Monaco has been giving increased priority to the use of nuclear techniques for the sustainable management of coastal zones — the parts of the ocean closest to human use and needs. Directions include laboratory and field-work on issues such as coastal water contamination, fishery concerns, harmful algae blooms, and land/ocean groundwater interactions. The IAEA also recently completed a thematic planning exercise on the 'Use of Nuclear and Isotopic Applications to Address Specific Coastal Zone Management Problems'. A copy of the framework document is available on request.

Focal points

Dr Thomas Tisue, Technical Co-operation Specialist, Department of Technical Co-operation, International Atomic Energy Agency, PO Box 100, Wagramerstrasse 5, A1190 Vienna, Austria
Tel.: +43 1-2600-26042
Fax: +43 1-2600-7
E-mail: T.Tisue@iaea.org
Website: http://www.iaea.org

Dr Florence Boisson, International Atomic Energy Agency, Marine Environment Laboratory, 4 Quai Antoine I^{er}, BP 800, MC 98012 Monaco Cedex, Principality of Monaco
Tel.: +377 97-97-72-64
Fax: +377 97-97-72-73
Website: http://www.iaea.org/monaco

Food and Agricultural Organization (FAO) of the United Nations

Background and activities

The FAO was founded in 1945 with a mandate to raise levels of nutrition and standards of living, to improve agricultural productivity, and to better the condition of rural populations. Today, it is one of the largest Specialized Agencies in the United Nations system and the lead agency for agriculture, forestry, fisheries and rural development. The FAO has 183 member countries plus one member organization, the European Community.

The *Codex Alimentarius* (food code) has become the seminal global reference point for consumers, food producers and processors, national food control agencies and the international food trade. The *Codex Alimentarius* system presents a unique opportunity for all countries to join the international community in formulating and harmonizing food standards and ensuring their global implementation. United Nations Resolution 39/248 advises that 'Governments should take into account the need of all consumers for food security and should support and, as far as possible, adopt standards from the… *Codex Alimentarius*' of the FAO and the World Health Organization.

The *Codex Alimentarius* has relevance for the international food trade. With respect to the ever-increasing global market, in particular, the advantages of having universally uniform food standards for the protection of consumers are self-evident. It is not surprising, therefore, that the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS) and the Agreement on Technical Barriers to Trade (TBT) both encourage the international harmonization of food standards. A product of the Uruguay Round of multinational trade negotiations, the SPS Agreement cites *Codex* standards, guidelines and recommendations as the preferred international measures for facilitating international trade in food. As such, *Codex* standards have become the benchmarks against which national food measures and regulations are evaluated within the legal parameters of the Uruguay Round Agreements. *Codex Alimentarius* includes guidelines on algal toxins, and is available at http://www.codexalimentarius.net/.

Liaisons

WHO-FAO *Codex Alimentarius* Commission; FAO-WHO Joint Expert Committee on Food Additives (JECFA).

Focal points

Mr Uwe Barg, Fishery Resources and Environment Division, Room NF 517, Vialle delle Terme di Caracalla, 00100 Rome, Italy
Tel.: +39 06-52253454
Fax: +39 06-52253020
Telex: 610181 FAO I
E-mail: Uwe.Barg@fao.org
Website: http://www.fao.org

World Health Organization (WHO)

Background

The World Health Organization is the United Nations agency with a specific mandate for the protection of public health. The agency's role in food safety is to protect the consumer against exposure to adverse effects from hazards in food. Article 2(u) of the WHO Constitution gives a mandate to develop, establish and promote international standards with respect to food. The organization has always recognized that access to adequate, nutritious, and safe food is a right of each individual. Its objective is the attainment by all peoples of the highest possible level of health, and an important prerequisite for health is safe food.

The International Programme on Chemical Safety (IPCS) was set up to provide assessment of the risks to human health and the environment from exposure to chemicals, whatever their origin, synthetic or natural. The Department of Protection of the Human Environment prepares guidelines in relation to safe drinking and recreational waters.

See section on the FAO for the WHO-FAO *Codex Alimentarius* Commission.

Goal

The International Programme on Chemical Safety (IPCS), established in 1980, is a joint programme of three co-operating organizations – International Labour Organization (ILO), United Nations Environment Programme (UNEP), WHO – implementing activities related to chemical safety. The WHO is the Executing Agency of the IPCS, whose main roles are to (a) establish the scientific basis for safe use of chemicals, and (b) strengthen national capabilities and capacities for chemical safety.

The WHO Food Safety Programme (FOS) was established in 1978. The goal of work in the food safety area is to reduce the burden of food-borne disease. To achieve this, the missions of the FOS are to (a) reduce the global burden of food-borne disease, (b) advocate a human health focus in food production and trade, (c) minimize the effect of new food-borne hazards, (d) provide a scientific basis for food safety standards, (e) raise the importance of food safety as a health issue, (f) increase countries' capacities to improve food safety.

Activities

In 1984 the IPCS published an Environmental Health Criteria document (No. 37) on *Aquatic (Marine and Freshwater) Biotoxins* covering toxicology of the most important biotoxins, such as paralytic shellfish poisons (PSP), ciguatera toxins, diarrhetic shellfish poisons (DSP), etc. IPCS-WHO publishes the series *Environmental Health Criteria* (EHC), and *Health and Safety Guides* (HSGs).

The WHO Department of Protection of the Human Environment is publishing *Guidelines for Drinking Water Quality, Guidelines for Safe Use of Recreational Waters*, and is developing *Water Quality Guidelines for Desalination*. All these publications address harmful algae or algal toxin issues.

The Food Safety Programme has been involved in food-borne illness caused by biotoxins. In 1981, a working group was convened on the public health aspects of marine food-fish poisoning. In 1984, the WHO published *Paralytic Shellfish Poisoning* (offset publication No. 79) to help prevent outbreaks of PSP. In 1989, a WHO consultation on public health aspects of seafood-borne zoonotic diseases

made several recommendations, including (a) the need to develop methods for monitoring seafood for algal toxins; (b) steps to harmonize tolerances for algae toxins in seafood and research on measures for preventing ciaguatera. In 1997, a Joint FAO/NACA/WHO study group on food safety issues associated with products from aquaculture concluded that there is a very small risk to human health associated with algal toxins in farmed finfish and crustaceans, which the group focused on. The FOS has published examples of poisonous fishes and crabs on the Internet (http://www.who.int/fsf/fish/index.html) together with illustrations and notes for the identification of those species.

Liaisons

FAO-WHO Joint Expert Committee on Food Additives (JECFA) and WHO-FAO *Codex Alimentarius* Commission.

Focal points

Dr J. Herrman, WHO Joint Secretary of JECFA, IPCS/World Health Organization, CH-1211 Geneva 27, Switzerland
Tel.: +41 22-791-3569
Fax: +41 22-791-4848
E-mail: herrmanj@who.int, http://www.who.int/pcs

Dr Jamie Bartram, World Health Organization, Water, Sanitation and Health, Department of Protection of the Human Environment, 20 Av. Appia, CH-1211 Geneva 27, Switzerland
Fax: +41 22-791-4159
E-mail: bartramj@who.int
Website: http://www.who.int/water_sanitation_health/

Hajime Toyofuku, Food Safety Programme, SDE/PHE, World Health Organization, 20 Av. Appia, CH-1211 Geneva 27, Switzerland
Office tel.: +41 22-791-3556
Tel.: +41 22-791-4807
E-mail: toyofukuh@who.ch
Website: http://www.who.int/fsf

International Maritime Organization (IMO)

Background

The International Maritime Organization is the United Nations Specialized Agency that develops and administers the international regulatory regime for maritime safety and the prevention of pollution from ships. The IMO's role may be summarized by the catchphrases 'Safer Ships – Cleaner Oceans' and 'Protecting Seafarers from the Sea – Protecting the Sea from Seafarers'. In addition to its Convention Secretariat role, the agency also provides technical assistance and co-operation to developing countries to assist in the implementation of its Conventions.

There is worldwide evidence that aquatic organisms have been translocated via ships' ballast water. This has resulted in significant environmental and human health problems. Many IMO Member States have expressed their concern on the introduction through ballast water discharges of toxic planktonic algae (primarily

dinoflagellates) in their coastal areas, resulting in human poisoning and fatalities from eating contaminated seafood, the periodic closure of shellfish farms, algal blooms and considerable loss of material income due to restricted exports. The IMO has responded to the ballast water problem by (a) forming a Ballast Water Working Group under its Marine Environment Protection Committee (MEPC); (b) adopting Guidelines for the control and management of ships' ballast water to minimize the transfer of harmful aquatic organisms and pathogens (Assembly Resolution A.868(20), hereafter referred to as the *IMO Guidelines*); (c) developing a new international legal instrument (Convention) on ballast water management (currently entitled International Convention for the Control and Management of Ships' Ballast Water and Sediments, hereafter referred to as the *Ballast Water Convention*), to be considered for adoption by an IMO Diplomatic Conference in late 2003; and (d) joining forces with the Global Environment Facility (GEF) and the United Nations Development Programme (UNDP) to assist developing countries to implement the *IMO Guidelines* and prepare for the *Ballast Water Convention*, through the Global Ballast Water Management Programme (GloBallast).

Goal

The overall goal of the IMO in this area is to develop and adopt a standardized international legal regime for the management of ships' ballast water, to minimize the transfer of harmful aquatic organisms and pathogens, including harmful algae, and to provide technical assistance to member countries to implement this regime.

Activities

IMO Guidelines

Until the new *Ballast Water Convention* is adopted, the *IMO Guidelines* should be used by governments and the shipping industry as the international standard for ballast water management. The *Guidelines* can be downloaded from the IMO website http://globallast.imo.org.

New Convention

The new *Ballast Water Convention* will provide a uniform, standardized, global ballast water management regulatory regime. The Convention is expected to be adopted by an IMO Diplomatic Conference in late 2003. The draft text of the Convention can also be found on the IMO website.

GloBallast Programme

In anticipation of the adoption of the new *Ballast Water Convention*, the IMO has also joined forces with the GEF and the UNDP to implement the Global Ballast Water Management Programme (GloBallast). The Development Objectives of this technical co-operation programme are to assist developing countries to reduce the transfer of harmful aquatic organisms and pathogens in ships' ballast water, to implement existing *IMO Guidelines*, and to prepare for the implementation of the *Ballast Water Convention*. Activities also include institutional strengthening and capacity building.

The website of the International Association of Independent Tanker Owners (INTERTANKO) provides profiles of national ballast water legislation, regulations and requirements (www.intertanko.com/tankerfacts/environmental/ballast/ballastreq.htm).

Liaisons

ICES-IOC-IMO Study Group on Ballast and Other Ship Vectors (SGBOSV); Global Invasive Species Programme (GISP); UNEP Regional Seas Programme and 'sister' GEF International Waters projects.

Focal points

Director, Marine Environment Division, International Maritime Organization, Marine Environment Division, 4 Albert Embankment, London SE1 7SR, UK
Tel.: +44 020-7735-7611
Fax: +44 020-7587-3210
Website: http://www.imo.org

International Council for the Exploration of the Sea (ICES)

Background

ICES is an international scientific organization studying and helping to safeguard North Atlantic marine ecosystems and the living resources they sustain. Founded in 1902, it is the oldest intergovernmental marine science organization in the world, and a leading forum for the promotion, co-ordination and dissemination of research on the physical, chemical and biological systems in the North Atlantic and advice on human impact on its environment, in particular the effects of fisheries in the North-east Atlantic. Since the 1970s, a major task for ICES has involved the provision of scientific information and advice in response to requests by international and regional regulatory commissions, the European Commission, and the governments of its member countries, for purposes of fisheries conservation and the protection of the marine environment. Because harmful algal blooms present problems quite distinct from those of normal blooms due to their potentially serious economic and social impacts, they require special attention. The organization plays a major role for its member countries in co-operating on problems relating to harmful algae because of its interdisciplinary capabilities.

Activities

The joint ICES-IOC Working Group on Harmful Algal Bloom Dynamics is the main focus on harmful algae, but related areas include the ICES Working Group on Phytoplankton Ecology, the Study Group on an ICES/IOC Checklist of Phytoplankton and other Protists, the Oceanography and Baltic Committees, and the Advisory Committee on the Marine Environment. A number of ICES members are active participants in GEOHAB and this is reflected in the development of other initiatives in ICES, which include the Study Group on GEOHAB Implementation in the Baltic, and the Study Group on Modelling of Physical/Biological Interactions, whose work included the development of models of harmful algal blooms.

Liaisons

Joint Working Group with IOC; ICES Working Group on Introductions and Transfers of Marine Organisms; ICES-IOC-IMO Study Group on Ballast and Other Ship Vectors (SGBOSV).

Focal points

ICES Secretariat, Harry Dooley, Palægade 2-4, DK-1261 Copenhagen K, Denmark
Tel.: +45 33-154225
Fax: +45 33-934215
E-mail: harry@ices.dk
Website: http://www.ices.dk/

INTERGOVERNMENTAL NON-SPECIALIZED ORGANIZATIONS

European Union, Commission of the European Communities (CEC)

Background

The CEC has a broad range of programmes, which includes, or potentially includes, projects on harmful and toxic algae. The specific focus may vary between the successive framework programmes.

EUROHAB is a cluster comprising EC/DG research projects on harmful algal blooms, which are formally linked together via a common attachment to the Technical Annex of each project.

EUROHAB, the European Initiative on Harmful Algal Blooms started in 1999, is formulated to generate and co-ordinate the required research to better manage the effects of toxic/harmful marine microalgae in the marine and brackish waters of the EU. The EUROHAB Science initiative was described in a publication of European Commission (European Commission, Research in Enclosed Seas-5, EUR 18592, ISBN 92-828-6612-2, 1999). The EUROHAB Initiative is an 'umbrella' that includes the environmental projects sponsored under FP-V on research for harmful algae. The European Commission/DG research is promoting today, through EUROHAB, both the high-level research and networking on HAB issues needed at European level and in co-ordination with relevant national activities.

Focal points

DG Research – EUROHAB
Dr Elisabeth Lipiatou, Environment and Sustainable Development Programme, Policy Aspects, European Commission, Directorate-General for Research
Mail: Rue de la Loi 200, B-1049 Brussels, Belgium
Office: Rue de Luxembourg 46, 1/75, B-1050 Brussels
Tel.: +32 02-2966286
Fax: +32 02-2963024
E-mail: Elisabeth.Lipiatou@cec.eu.int

NON-GOVERNMENTAL ORGANIZATIONS

International Society for the Study of Harmful Algae (ISSHA)

Background

ISSHA was founded in 1997, in response to a request from the Intergovernmental Oceanographic Commission (IOC) of UNESCO for an international programme on harmful algae. The society is affiliated to the ICSU family of organizations as a constituent part of the International Union of Biological Sciences (IUBS) through the International Association of Biological Oceanography (IABO). The aim of the society is to advance understanding by the promotion and pursuit of all aspects of the study of harmful algae and to widely disseminate the results of this research.

Activities

Activities include the promotion of the study of harmful algae, including their occurrence, related oceanographic factors, ecophysiology, taxonomy and systematics, genetics, toxin chemistry, toxicology, management and mitigation; the collection, evaluation and dissemination of information on harmful algae; the promotion of harmful algal research, training and extension for fostering the related management and mitigation; the development of material for raising public awareness of the social, economic and ecological effects caused by harmful algae; the organization and co-sponsorship of national, regional and international conferences, seminars, symposia and working group meetings; the organization of lectures and courses; the publication of scientific and popular articles; and the support and implementation of research projects and programmes linked to the study of harmful algae.

Focal points

President: Karen Steidinger, Florida Marine Research Institute, 100 8th Ave. S.E., St Petersburg, FL 33712, USA
Fax: +1 727-823-0166, karen.steidinger@fwc.state.fl.us

Secretary: Stephen Bates, Fisheries and Oceans Canada, Gulf Fisheries Centre, PO Box 5030, Moncton, NB E1C 9B6, Canada
Fax: +1 506-851-2079
E-mail: BatesS@mar.dfo-mpo.gc.ca
Website: http://www.cbr.nrc.ca/issha/

International Society for Toxinology (IST)

Background

The purpose of the society is to advance knowledge on the properties of toxins and antitoxins and to bring together scholars interested in these substances. The IST has 600 members in 57 countries.

Goal

One of the society's goals is research on toxic algae, including red tides, ciguatera poisoning, etc. An important aspect is exchange of results in meetings, such as satellite symposia on marine pharmacology.

Activities

Major international meetings are held regularly, and the society edits its own journal *Toxicon* (founded 1962).

Liaisons

Joint activities with WHO, ESF, ISF and state institutions in the countries of its members.

Focal points

President: Prof. Dr Hervé Rochat, Faculté de Médicine, Sécteur Nord, Biochimie, Boulevard Pierre Dramard, F-13916 Marseille Cedex 20, France
Tel.: +33 4-91-51-28-48
Fax: +33 4-91-65-75-95
E-mail: rochat.h@jean-roch.univ.mrs.fr

Secretary: Prof. Dr Dietrich Mebs, Zentrum der Rechtsmedizin, University of Frankfurt, Kennedyallee 104, D-60596 Frankfurt, Germany
Tel.: +49 69-63-01-75-63
Fax: +49 69-63-01-58-82
E-mail: mebs@em.uni-frankfurt.de

Association of Official Analytical Chemists (AOAC)
Methods Committee on Natural Toxins

Background

The AOAC Official Methods Programme co-ordinates the development and validation of chemical and microbiological analytical methods by expert scientists working in their government, industry and academic laboratories worldwide. Candidates for AOAC Official Methods status are subjected to a collaborative study involving a minimum of eight laboratories and conducted according to an internationally harmonized and recognized protocol. The proposed methods are reviewed first by an expert (General Referee), statistical and safety advisors, and then a broader committee of experts, the Methods Committee on Natural Toxins. Adopted methods are published in the *Journal of AOAC International*. The Methods Committee on Natural Toxins is responsible for guiding and co-ordinating the development and validation of analytical methods for the detection, identification, and/or quantization of mycotoxins, plant toxins and seafood toxins in food. Those methods demonstrating sufficient accuracy, precision and specificity will be recommended for adoption by AOAC International as AOAC Official Methods.

Goal

To provide methods of analysis for natural toxins for which performance characteristics such as accuracy, precision, sensitivity, range, specificity and similar attributes have been determined and tested under typical laboratory applications.

Activities

Review and recommend General Referee topics, such as seafood toxins, and Associate Referee topics such as ciguatera, cyanobacterial peptide toxins, domoic acid, and diarrhetic, neurotoxic and paralytic shellfish poisoning toxins. Review proposed

methods and collaborative study protocols, such as Solid Phase Immunobead Assay Method for Ciguatoxins in Fish, Liquid Chromatographic Method for Domoic Acid in Shellfish, etc. Publish General Referee reports, summarizing activities and literature for the year, in the *AOAC Journal*.

Sponsor special-interest symposia at the AOAC International annual meeting, such as Analytical Methods for Seafood Toxins. Publish peer-reviewed reports of research in the *Journal of AOAC International*, such as comparisons of mouse bioassays and cell bioassays for ciguatoxins; liquid chromatographic methods for domoic acid; detection of marine toxins using reconstituted sodium channels; detection methods based on radioimmunoassay; and pre-column oxidation techniques for determining saxitoxin.

Liaisons

Joint activities with IUPAC Environmental Chemistry Division; International Standards Organization; Royal Society of Chemistry Analytical Methods Committee; Nordic Committee for Food Analysis, Codex Committee for Methods of Analysis and Sampling; and numerous others.

Focal points

AOAC staff

A. E. Pohland, Director, Office of International Activities, AOAC International, 481 N. Frederick Ave., Suite 500, Gaithersburg, MD 20877-2417, USA
Tel.: +1 310-924-7077 Ext. 123
Fax: +1 301-924-7089
E-mail: apohland@aoac.org

Member leadership

Michael A. Quilliam, General Referee on Phycotoxins, Institute for Marine Biosciences, National Research Council of Canada, 1411 Oxford Street, Halifax, NS B3H 3Z1, Canada
Tel.: +1-902-426-9736
Fax: +1 902-426-9413
E-mail: michael.quilliam@nrc.ca
Website: http://www.aoac.org

Scientific Committee on Oceanic Research (SCOR)

Background

Recognition that scientific questions about the ocean often require an interdisciplinary approach led the International Council for Science (ICSU) to form the Scientific Committee on Oceanic Research (SCOR) in 1957. The activities of SCOR, the first interdisciplinary body formed by ISCU, focus on promoting international cooperation in planning and conducting oceanographic research, and solving methodological and conceptual problems that hinder research. Scientists from thirty-six SCOR member nations participate in working groups and steering committees.

As part of its mandate to promote international collaboration in marine science, SCOR establishes small working groups with terms of reference that normally are accomplished within three to four years. The committee also co-sponsors, with other international organizations, major ocean research programmes. SCOR has identified

harmful algal blooms as a scientific topic in which progress could be made through its activities. An initial effort on this topic was the SCOR-IOC Working Group on the Physiological Ecology of Harmful Algal Blooms, WG 97, which worked in close co-operation with the IOC Harmful Algal Bloom Programme and the ICES-IOC Working Group on Harmful Algal Bloom Dynamics.

Activities

WG 97 recommended the creation of a major research programme on the Global Ecology and Oceanography of Harmful Algal Blooms (GEOHAB), which is developing research collaboration between SCOR and IOC.

Liaisons

Joint research programme (GEOHAB) with IOC.

Focal points

SCOR Secretariat, Department of Earth and Planetary Sciences, Johns Hopkins University, Baltimore, MD 21218, USA
Tel.: +1 410-516-4070
Fax: +1 410 516-4019
E-mail: scor@jhu.edu
Website: http://www.jhu.edu/~scor

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