1 Introduction to methods for quantitative phytoplankton analysis

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Background

Phytoplankton is a critical component of the marine ecosystem as they are responsible for approximately half of the global (terrestrial and marine) net primary production (Field *et al*. 1998). Today approximately 4000 marine phytoplankton species have been described (Simon *et al.* 2009). They have the potential to serve as indicators of hydro-climatic change resulting from global warming as well as other environmental impacts, such as ocean acidification due to combustion of fossil fuels and eutrophication. Under certain environmental conditions phytoplankton can experience elevated growth rates and attain high cell densities. This is known as an algal bloom. There are different types of algal blooms. Some are natural events such as the spring diatom bloom where, at temperate latitudes, there is a burst of diatom growth during spring time as a response to increasing light availability, temperature and water column stabilisation. This is part of the annual phytoplankton cycle in these regions. Some blooms can have a negative impact on the marine system and aquaculture industry and are termed 'Harmful Algal Blooms' (HABs). Some HAB species such as the dinoflagellate, *Karenia mikimotoi,* form high density blooms with millions of cells *per* Litre discolouring the water and causing anoxia as the bloom dies off. This can result in benthic mortalities such as starfish, lugworms and fish. In contrast, low cell densities of species of the dinoflagellate genus *Alexandrium (*2,000 cells L^{-1}) have been associated with closures of shellfish harvesting areas owing to elevated levels of the toxins responsible for paralytic shellfish poisoning. These are also called HABs even though they are present at low cell densities.

Many regions of the world implement phytoplankton monitoring programmes to protect their aquaculture industry. These programmes provide advice about the potential for toxic events and improve local knowledge of the dynamics of toxic phytoplankton in the area. The European Union (EU) member states are legally obliged to monitor their shellfish production areas for the presence of toxin producing phytoplankton. Marine environmental policy has increased in importance and a number of directives has been developed to monitor water quality. The Water Framework Directive (WFD) uses phytoplankton as one of the ecosystem components required to monitor the quality status of marine and freshwater bodies. Phytoplankton is also a required biological component of the EU Marine Strategy Framework Directive, devised to protect and conserve the marine environment. The International Maritime Organization (IMO) adopted the Ballast Water Convention in 2004 although it has not yet been ratified. This convention includes a ballast water discharge standard whereby ships will be required to treat or manage ballast water to ensure that no more than 10 organisms *per* mL in the size category >10 µm - < 50 µm and no more than 10 organisms per m^3 >50 µm are discharged.

Thus, there is a requirement to be able to describe and monitor the abundance, composition and diversity of the phytoplankton community. A variety of different methods have been developed to identify and enumerate phytoplankton. Descriptions of many of these can be found in two UNESCO-produced volumes: The *Phytoplankton manual*, edited by Sournia, was published in 1978. This volume provides a comprehensive description of many traditional light microscopy methods used to enumerate phytoplankton. It is currently out of print and many laboratories have found it difficult to obtain a copy. The *Manual on Harmful Marine Microalgae* edited by Hallegraeff *et al.* was first published in 1995 with a revised second edition published in 2004. It provides information on the taxonomy and methodology involved in operating phytoplankton and biotoxin monitoring programmes.

The present manual aims to provide detailed step by step guides on how to use microscope based and molecular methods for phytoplankton analysis. Most of the molecular methods are aimed only at selected target species while some of the microscope based methods can be used for a large part of the phytoplankton community. Methods for analyzing autotrophic picoplankton are not included in this manual. Common methods for this important group include fluorescence microscopy (Platt and Li 1986 and references therein) and flow cytometry (e.g. Simon *et al* 1994) as well as molecular methods. The decision on which method to use will ultimately depend on the purpose of the monitoring programme and the facilities and resource available. Information about sampling strategies are found in Franks and Keafer (2004). Although the sampling methods are outside the scope of this manual an overview of the steps from sampling to presentation of results to end users is presented in Fig. 1. Examples of sampling devices are found in Figs. 2-7. In addition to these automated sampling systems on Ships of Opportunity (SOOP, e.g. FerryBox systems), buoys, Autonomous Underwater Vehicles (AUV's) etc. are used (Babin *et al*. 2008).

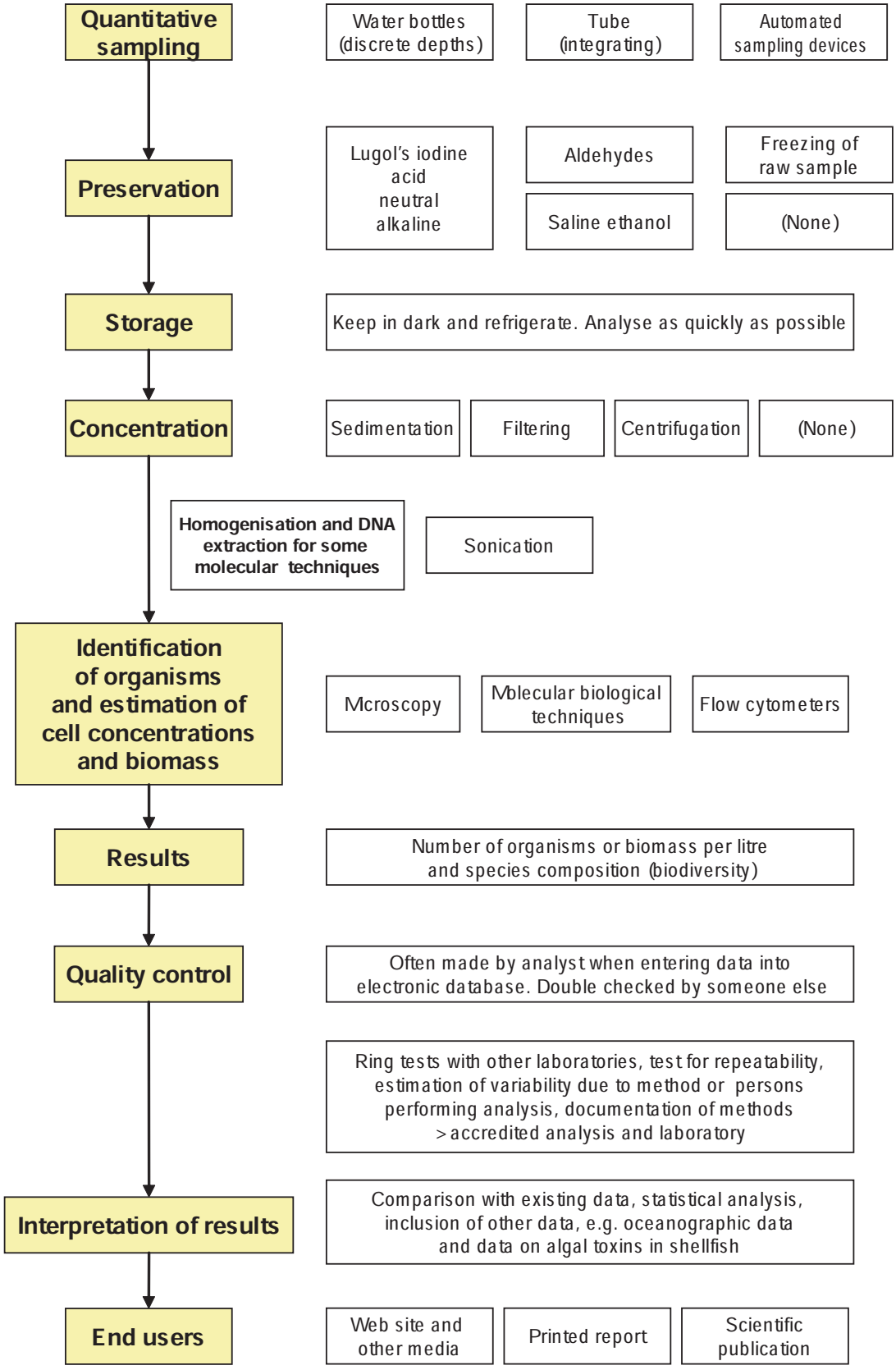

Figure 1. Schematic drawing of the steps from sampling to results.

Table 1. Examples of web sites that provide useful information for phytoplankton analysts.

Species information	**URL**
AlgaeBase	www.algaebase.org
World Register of Marine Species, WoRMS	www.marinespecies.org
IOC-UNESCO Taxonomic Reference List of Harmful Micro Algae	www.marinespecies.org/hab/index.php
European Register of Marine Species, ERMS	www.marbef.org
Integrated Taxonomic Information System, ITIS	www.itis.gov
Micro*scope	starcentral.mbl.edu/microscope/
Plankton*net	www.planktonnet.eu
Encyclopedia of Life	www.eol.org
Gene sequences etc.	
Genbank	www.ncbi.nlm.nih.gov/Genbank/
European Molecular Biological Laboratory	www.embl.org
National Center for Biotechnology Information	www.ncbi.nlm.nih.gov

Microscopy based techniques

The historical development of microscope based phytoplankton analysis techniques

Many historic reports exist of phytoplankton blooms. Some believe the description of the Nile water changing to blood in the bible and resultant fish mortalities (Exodus 7:14-25) is an account of the occurrence of a HAB. The invention of the microscope by Anton van Leeuwenhoek (1632-1723) in the 17th century allowed more detailed observations of phytoplankton to be made with Christian Gottfried Ehrenberg (1795-1876) and Ernst Heinrich Philipp August Haeckel (1834-1919) becoming pioneers in observations of microalgae. Over the last 150 years a number of techniques for analysis of phytoplankton have been developed and adopted in analytical laboratories throughout the world. The Swedish chemist, Per Teodor Cleve (1840-1905), was one of the first researchers to undertake more quantitative surveys of the phytoplankton community. He used silk plankton nets to investigate the distribution of phytoplankton in the North Sea Skagerrak-Kattegat area (1897). Hans Lohmann (1863-1934) first used a centrifuge to concentrate plankton and discovered the nanoplankton (phytoplankton 2 – 20 µm in size) (Lohmann 1911). The classic sedimentation chamber technique still used in many laboratories today was developed by Utermöhl (1931, 1958). In the 1970s the fluorescence microscope was first used for quantitative analysis of bacteria in seawater (e.g. Hobbie *et al.* 1977). A similar technique was used to reveal the ubiquitous distribution of autotrophic picoplankton (size 0.2 – 2 µm) in the sea (Johnson and Sieburth 1979, Waterbury *et al.* 1979). In the 1980s auto- and heterotrophic nanoplankton were investigated using various stains and filtration techniques (e.g. Caron 1983).

Training and literature for identification of phytoplankton using microscopes

Microscope based methods involve the identification of phytoplankton species based on morphological and other visible criteria. Phytoplankton taxonomists should have a high degree of skill and experience in the identification of the species present in their waters and appropriate training should be incorporated into their work programme. Access to key literature for phytoplankton identification, such as Horner (2002), Tomas (1997) and Throndsen *et al.* (2003, 2007) is essential. Access to older scientific literature is often necessary for detailed species descriptions, however, these may be difficult to access. Attendance at phytoplankton identification training courses when possible is the most successful way to allow analysts to continue to learn and develop their skills. This is especially important since the systematics and nomenclature of phytoplankton is constantly under revision. Species lists and images of phytoplankton are presented in a variety of web sites, see examples listed in Table 1. While a wealth of information is available on the internet, they cannot replace teaching and guidance from an experienced taxonomist.

Microscopes for phytoplankton identification and enumeration

A high quality microscope is essential for the enumeration and identification of phytoplankton species. Although the initial cost will be high, a microscope, if serviced on a regular basis, can remain in use for many years. Two types of microscopes are commonly used: (1) the standard compound (upright) microscope and (2) the inverted microscope (Figs. 8 - 9). With the inverted microscope, the objectives are positioned underneath the stage holding the sample. This is necessary for examination of samples in sedimentation chambers and flasks where the phytoplankton cells have settled onto the bottom. Oculars should be fitted with a graticule and a stage micrometer is used to determine and calibrate the length of the scale bars of the eyepiece graticule under each objective magnification. In Fig. 10 examples of how *Alexandrium fundyense* is viewed in the microscope using different micrsocope and staining techniques are presented. The digital photographs were taken during a workshop comparing micrsocopic a and molecular biological techniques for quantiative phytoplankton analysis. Results from the workshop are found in Godhe *et al.* (2007).

Because many phytoplankton species are partially transparent when viewed under a light microscope, different tech-

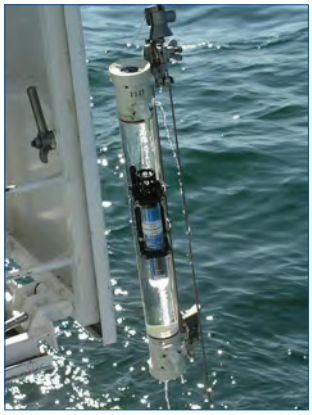

Figure 2. Reversing water sampler of the modified Nansen type.

Figure 3. Water sampler of the Ruttner type.

Figure 4. CTD with rosette and Niskin-type water bottles. An *in situ* chlorophyll a fluorometer is also mounted.

Figure 5. Phytoplankton net. This is not used for quantitative sampling but for collecting rare, non fragile species.

Figure 6. Tube for integrated water sampling.

Figure 7. The Continuous Plankton Recorder. This device is mainly aimed for sampling zooplankton but may be useful for collecting larger, non fragile phytoplankton species. Photo courtesy of the Sir Alister Hardy Foundation for Ocean Science, SAHFOS http://www.sahfos.ac.uk/.

niques to improve contrast are used. Differential Interference Contrast (DIC, also called Normarski) and Phase Contrast are popular. DIC is considered by many to be the optimal method for general phytoplankton analysis. Most plastic containers, however, cannot be used with this method as many plastics depolarize the required polarized light. It is also more expensive than Phase Contrast and requires a different set of objectives, polarizing filters etc. to function properly.

Natural fluorescence

Fluorescence generated from photosynthetic and other pigments in phytoplankton can be used as an aid for the identification and enumeration of species. This works best with live samples and samples preserved with formaldehyde or glutaraldehyde. If Lugols iodine is used for preservation, the natural fluorescence is not visble. Fluorescence can also be used to differentiate between heterotrophic and autotrophic organisms. The microscope must be equipped with objectives suitable for fluorescence, a lamp housing for fluorescence (e.g. mercury lamp 50 or 100 W), the required filter sets. A useful filter set to observe fluorescence from both chlorophyll *a* and phycoerythrin consists of a filter for excitation at 450-490 nm and a long pass filter for emission at 515 nm.

Staining of cells

Different stains are used to aid the identification of phytoplankton species. In this volume only fluorescent stains (fluorochromes) are discussed. The stain used in chapters 2 and 5, calcofluor, binds to the cellulose theca in armoured dinoflagellates and allows a detailed examination of the plate structure to be performed. This stain is very useful when morphologically similar species, e.g. *Alexandrium* spp., are present. Fluorochromes are also often used in connection with antibodies or RNA targeted probes to identify phytoplankton. Some of these are covered in chapter 9. It should be noted that some microscope objective lenses do not transmit ultraviolet light and are unsuitable for work with fluorochromes that require UV-light excitation, e.g. calcofluor.

Image analysis

Manual phytoplankton analysis with microscopy may be time consuming and analysts must possess the necessary skills to allow the identification of cells using morphological features. This has led to interest in the use of automated image analysis of phytoplankton samples. Basic image analysis methods do not generally discriminate between phytoplankton and other material such as detritus and sediment in samples thereby presenting a problem in the application to routine field samples. This technique may be more useful for the analysis of cultures and monospecific high density blooms. Researchers have tried more advanced methods such as artificial neural networks (ANN) to identify species automatically by pattern recognition. Some ANN software includes functions which train the ANN to identify certain species. One such instrument under development is the HAB Buoy, which uses the Dinoflagellate Categorisation by Artificial Neural Network (DICANN) recognition system software (Culverhouse *et al.* 2006). Other examples of software currently under evaluation for automated phytoplankton identification are used in Flow Cytometers (see next paragraph), e.g. the FlowCAM (chapter 8) and the method described by Sosik and Olson (2007). To date, these methods require a highly trained phytoplankton identification specialist to train the software to recognise the images and carry out a quality control on the results of the automated image analysis.

Flow cytometry

A flow cytometer is a type of particle counter initially developed for use in medical science. Today instruments have been developed for use specifically in aquatic sciences. Autofluorescence and scattering properties are used to discriminate different types of phytoplankton. The different phytoplankton groups are in general not well distinguished taxonomically when a standard instrument is used. A standard flow cytometer is very useful to estimate abundance of e.g. autotrophic picoplankton. A more advanced type of flow cytometer has a camera that produces images of each particle/organism. Automated image analysis makes it possible to identify organisms. Manual inspection of images by an experienced phytoplankton identification specialist is required for quality control and for training the automated image analysis system. A desk top system is described in chapter 8. An example of an *in situ* system is described by Sosik and Olsen (2007) and Olsen and Sosik (2007).

Molecular techniques

Significance of molecular based phytoplankton analysis techniques

Owing to some of the difficulties and limitations of morphological identification techniques, microalgal studies are increasingly exploring the use of molecular methods. Most molecular techniques have their origin in the medical science, and during the last three decades these various techniques have been tested, modified, and refined for the use in algal identification, detection and quantification.

The development of molecular tools for the identification and detection of microalgae has influenced and improved other fields of phycological research. Molecular data are gaining influence when the systematic position of an organism is established. Today, the description of new species, erection of new genera, or rearrangement of a species to a different genus is usually supported by molecular data in addition to morphological structures, ultrastructure, and information on biogeographic distribution (e.g. Fraga *et al.* 2008). Thus, the understanding of evolutionary relationships among microalgal taxa has been immensely improved (Saldarriaga *et al.* 2001). Spatially separated populations of microalgal species might display different properties, such as toxin production. By studying minor differences within the genome, populations can be confined to certain locations, and human assisted and/or natural migration of populations can be investigated (e.g. Persich *et al.* 2006, Nagai *et al.* 2007). Also, the increasing information on the structure of genes and new tools for investigating their expressions, have enhanced our understanding of algal physiological processes (Maheswari *et al.* 2009).

Laboratory requirements for molecular techniques

Different types of molecular techniques have very different requirements for laboratory facilities and instruments. The range is from very well equipped laboratories to field instruments. In chapters 9-14 examples of laboratory methods are

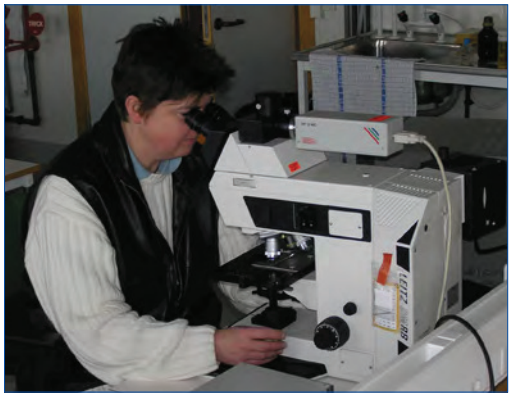

Figure 8 Compound microscope

Figure 9. Inverted microscope

found. *In situ* systems are under development (e.g. Paul *et al.* 2007 and Scholin *et al.* 2009).

Identification and quantification of phytoplankton species by molecular methods

Molecular methodologies aim to move away from species identification and classification using morphological characteristics that often require highly specialist equipment such as electron microscopes, or very skilled techniques such as single cell dissections. Instead molecular techniques exploit differences between species at a genetic level. Molecular analysis requires the use of specialised equipment and personnel and most importantly requires a previous knowledge of the genetic diversity of the phytoplankton in a specific region. To date, molecular methods have been used to support HAB monitoring programmes in New Zealand and the USA (Rhodes *et al.* 1998, Scholin *et al.* 2000, Bowers *et al.* 2006).

In this present manual, methods based on ribosomal RNA (rRNA) and DNA (rDNA) targeted oligonucleotides and polymerase chain reaction (PCR) are described. Oligonucleotides and PCR primers are short strains of synthetic RNA or DNA that is complementary to the target RNA/DNA. Molecular sequencing of phytoplankton cells has generated DNA sequence information from many species around the world. This has allowed the design of oligonucleotide probes and PCR primers for specific microalgal species. Some oligonucleotide probes, which hybridize with complementary target rRNA or rDNA, have a fluorescent tag attached and can act as a direct detection method using fluorescence microscopy. PCR primers enable the amplification of target genes through PCR. The primers serve as start and end points for *in vitro* DNA synthesis, which is catalysed by a DNA polymerase. The PCR consists of repetitive cycles, where in the first step, DNA is heated in order to separate the two strands in the DNA helix. In the second step during cooling, the primers, which are present in large excess, are allowed to hybridize with the complementary DNA. In a third step, the DNA polymerase and the four deoxyribonucleoside triphosphates (dNTPs) complete extension of a complementary DNA strand downstream from the primer site. For effective DNA amplification, the three steps are repeated in 20-35 cycles (Alberts *et al.* 1989). A useful volume covering the basics of molecular methods and general applications is Molecular Systematics edited by Hillis *et al.* (1996).

Most of the molecular methods described here, with the exception of the whole cell assay (chapter 9 and 14), do not require the cells to remain intact. In these methods the rRNA molecules in the cell's cytoplasm or the nuclear DNA are released during nucleic acid extraction and are targeted by the probes or PCR primers. During the whole cell assay, the target rRNA/rDNA within intact cells is labelled with fluorescently tagged probes. It is therefore vital that the laboratory protocol used ensures that the probes can penetrate the cell wall in order to access target genetic region and label them. Tyramide Signal Amplification has been used with FISH (TSA-FISH) to further enhance fluorescence signals (see chapter 14). The fluorescent tag can then be read using a fluorescent microscope as with the whole cell assays (FISH chapter 9) or additional technology is employed to allow these fluorescent tags to be read automatically e.g. using a sandwich hybridization technique (chapter 12) and PCR (chapter 13).

The hand held device and DNA-biosensor with disposable sensorchip (sandwich hybridisation, electrochemical detection) and DNA microarray technology (fluorescent detection) methods discussed in this manual are still at the final development stages (see chapters 10 and 11). Within the next decade these methods may be ready to be incorporated into monitoring programmes. The authors suggest that future advances in this field will include microarray/DNA chip (sometimes called "phylochips") technologies with probes for multiple species applied *in situ* to an environmental sample simultaneously.

Alternative molecular based methods such as lectin (protein and sugar) binding and antibody based assays (e.g. immunofluorescence assays) are not included in this manual. Information on these molecular diagnostic tools may be found in chapter 5 of The Manual on Harmful Marine Microalgae (Hallegraeff *et al.* 2004).

Molecular method validation

rDNA and rRNA have become the most popular target regions for microalgal species identification. These regions are attractive for primer and probe design because they contain both conserved and variable regions and are ubiquitous in

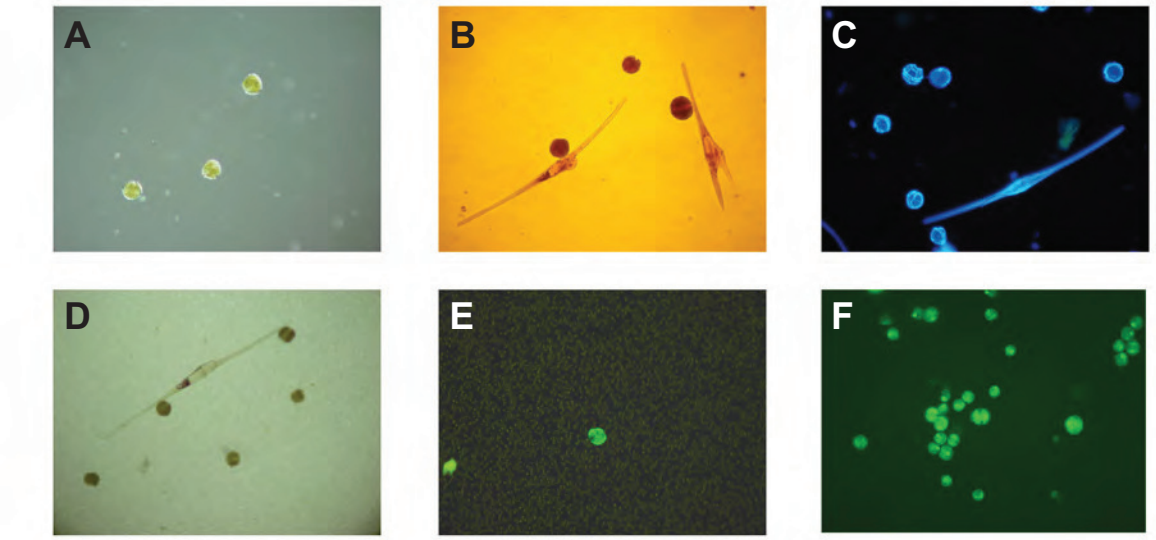

Figure 10. *Alexandrium fundyense* as seen in the microscope using different techniques. *Ceratium* spp. are present in B, C and D. A. Filter freeze transfer with contrast enhancement using DIC (appearance in Utermöhl is essentially identical), B. Sedimentation flask, C. Filtering + calcofluor staining, D. Filtering using semitransparent filters, E.and F. Whole cell hybridization assay.
Sample preparation: A. Allan Cembella, B. Georgina McDermott, C. Per Andersen, D. Einar Dahl, E. Melissa Gladstone and F. David Kulis. F is originally a greyscale image to which artificial colour has been added to simulate what the eye sees. Photo A-E Bengt Karlson and F David Kulis. Microscope. A-E Zeiss Axiovert 200 and F Zeiss Axioplan 40 Fl.

all organisms. In addition, a large number of sequences are available in molecular web based databases, e.g. GENBANK, for sequence comparative analyses (Table 1) and design of oligonucleotide probes and PCR primers. Despite extensive sequence analysis of cultured phytoplankton species, cross reactivity with other organisms in the wild may occur, it is therefore crucial to test the developed probes/primers with the target species and several non-target species. Method development, although time consuming, is essential if these methods are to be implemented. It is the responsibility of the end user to ensure that specificity to the target organism is evaluated appropriately.

Quality control

As with all scientific research, it is necessary to investigate the variability of the methods used before employment into any monitoring programme. The variability of the result can be affected by cell abundance which can dictate the method of choice. Further information on this can be found in chapter 2 and of Venrick (1978 a,b,c) and Andersen and Throndsen (2004). Many laboratories have achieved national accreditation for techniques described in this manual. This involves developing protocols with levels of traceability and reproducibility in line with defined criteria. Participation in internationally recognised inter-laboratory comparisons are strongly recommended.

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2 The Utermöhl method for quantitative phytoplankton analysis

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Introduction

The Utermöhl method (Utermöhl 1931, 1958) has an advantage over other methods of phytoplankton analysis in that algal cells can be both identified and enumerated. Using this method, it is also possible to determine individual cell size, form, biovolume and resting stage.

The Utermöhl method is based on the assumption that cells are poisson distributed in the counting chamber. The method is based on the sedimentation of an aliquot of a water sample in a chamber. Gravity causes the phytoplankton cells to settle on the bottom of the chamber. The settled phytoplankton cells can then be identified and enumerated using an inverted microscope. To quantify the result as cells per Litre a conversion factor must be determined.

Materials

Equipment

Sample Bottles

If samples are analysed immediately or within a few days plastic vials may be used. Note that the preservatives may be absorbed by the plastic. For long term storage, glass sample bottles should be used to minimise any chemical reaction with the preservative. Clear glass bottles allow the state of Lugol's iodine preservation to be easily monitored (Fig. 1). These samples must be stored in the dark to prevent the degradation of Lugol's iodine in light. It is important that the bottle cap is securely tightened to avoid spillage of the sample and evaporation of the preservative. Utermöhl (1958) recommended that the bottle is filled to 75-80% of its volume. This facilitates the homogenisation of the sample before dispensing into the sedimentation chamber.

Preservation agents

Preservation agents must be chosen depending on the objective of the study. The most commonly used is potassium iodine; Lugol's iodine solution – acidic, neutral or alkaline (Table 1; Andersen and Throndsen 2004). If samples are stored for long periods they may be preserved with neutral formaldehyde (Table 2).

Table 2. Recipe for neutral formaldehyde. (from: Throndsen 1978, Edler 1979, Andersen and Throndsen 2004). Filter after one week to remove any precipitates.

Neutral formaldehyde
500 mL 40% formaldehyde
500 mL distilled water
100 g hexamethylentetramid
pH 7.3 – 7.9

Sedimentation chambers

The sedimentation chamber consists of two parts, an upper cylinder (chimney) and a bottom plate with a thin glass (Fig. 2). They are usually made of perspex in volumes of 2, 5, 10, 25 or 50 mL. The thickness of the glass base plate should not exceed 0.2 mm, as this will affect the resolution achievable by the microscope. Counting chambers should be calibrated. This is achieved by first weighing the chamber while empty and then filled with water to confirm the volume.

The inverted microscope

For quantitative analysis using sedimentation chambers, an inverted microscope is required (Fig. 3). The optical quality of the microscope is crucial for facilitating phytoplankton identification. Phase- and/or differential interference-contrast is helpful for the identification of most phytoplankton, whereas bright-field may be advantageous for coccolithophorids (Heimdahl 1978).

Epifluorescence equipment is a great advantage for counting and identification of organisms with cellulose cell walls, e.g., thecate dinoflagellates, chlorophytes and "fungi". A stain is applied to the sample which causes cellulose to fluoresce.

One eyepiece should be equipped with a calibrated ocular micrometer. The other eyepiece should be equipped with two parallel threads forming a transect. A third thread perpendicular to the other two facilitates the counting procedure (Fig. 4 a). It is also possible to have the eyepiece equipped with other graticules such as a square field or grids (Fig. 4 b). The eyepiece micrometer and counting graticule must be calibrated for each magnification using a stage micrometer.

Table 1. Recipes for Lugol's iodine solution (acidic, alkaline and neutral). (from: Utermöhl 1958, Willén 1962, Andersen and Throndsen, 2004).

Acidic	Alkaline	Neutral
20 g potassium iodide (KI)	20 g potassium iodide (KI)	20 g potassium iodide (KI)
10 g iodine (I_2)	10 g iodine (I_2)	10 g iodine (I_2)
20 g conc. acetic acid	50 g sodium acetate	200 mL distilled water
200 mL distilled water	200 mL distilled water	

The fundamentals of

The Utermöhl method

Scope
Qualitative and quantitative analysis of phytoplankton.

Detection range
Detection range is dependent on the volume of sample settled. Counting all of the cells in a 50 mL chamber will give a detection limit of 20 cells per Litre.

Advantages
Qualitative as well as quantitative analysis. Identification and quantification of muliple or single species. Detection of harmful species.

Drawbacks
This is a time consuming analysis that requires skilled personnel. Sedimentation time prevents the immediate analysis of samples. Autotrophic picoplankton is not analysed using the Utermöhl method.

Type of training needed
Analysis requires continuous training over years with in-depth knowledge of taxonomic literature.

Essential Equipment
Inverted microscope, sedimentation chambers, microscope camera, identification literature, (epifluorescense equipment, counting programme).

Equipment cost*
Inverted microscope: 7,500 – 50,000 € (11,000 – 70,000 US $).
Sedimentation chamber: 150 € (200 US$).
Microscope camera: 3,000 – 8,000 € (4,300 – 11,000 US $).
Identification literature: 1,000 – 3,000 € (1,400 – 4,300 US $).
Epifluorescense equipment: 10,000 € (14,000 US $).
Counting programme: 500 – 5,000 € (700 – 7,000 US $).

Consumables, cost per sample*
Less than 5 €/4 US $.

Processing time per sample before analysis
App. 10 minutres for filling and assembling sedimentation chamber.
3-24 hours sedimtation time depending on volume and analysis type.

Analysis time per sample
2-10 hours or more depending on type of sample and analysis.

Sample throughput per person per day
1-4 depending on type of sample and analysis.

No. of samples processed in parallel
One per analyst.

Health and Safety issues
Analysis sitting at the microscope is tiresome for eyes, neck and shoulder. Frequent breaks are needed. If formalin is used as preservation agent appropriate health and safety guidelines must be followed.

*service contracts not included
**salaries not included

Figure 1. Sample bottles: glass and plastic. Bottle of Lugol's iodine solution to the right.

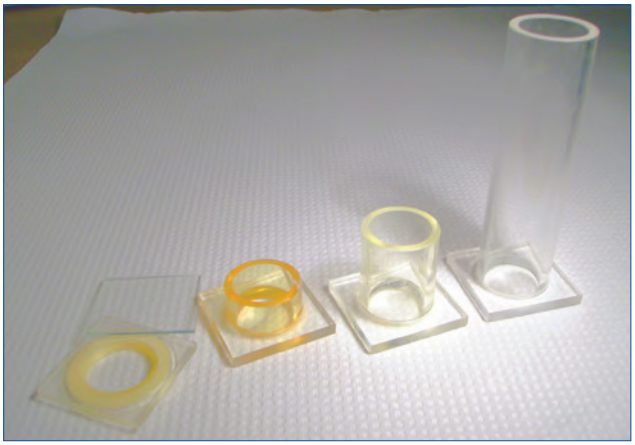

Figure 2. Sedimentation chambers. From left to right: bottom plate with cover glass, 10 mL chamber, 25 mL chamber and 50 mL chamber.

Figure 3. Inverted microscope.

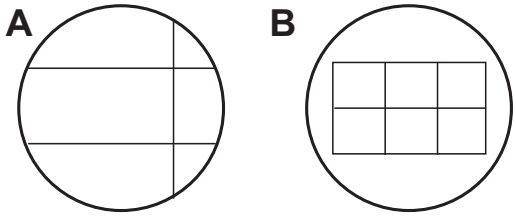

Figure 4. Counting aids mounted in the eyepiece. a) parallel threads, with a transverse thread. b) grids.

The microscope should have objectives of 4-6X, 10X, 20X and 40-60X. For detailed examination a 100X oil immersion objective may also be used. If epifluorescence microscopy is to be used, the microscope must be equipped with the appropriate objective lenses. In order to survey the entire bottom plate the microscope must be equipped with a movable mechanical stage.

Cell counters

A cell counter with 12 or more keys is a useful device. Medical blood cell counters (Fig. 5) are commonly used. If these are not availabe single tally counters can be used as appropriate. It is also common to have a computerised counting programme (Fig. 6) beside the microscope, so that the observed species are registered directly into a database.

Laboratory facilities

Laboratory facilities necessary for the quantitative analysis of phytoplankton require amenities for storing, handling (mixing and pouring samples) and washing of sedimentation chambers. Preserved samples should be stored in cool and dark conditions. During sedimentation the chambers should be placed on a level, horizontal and solid surface. This will prevent any non random accumulation of phytoplankton cells.

Methods

Preparation of sample

Preservation

Once the sample has been collected from the field and poured into the sample bottle it should be immediately preserved using either:

Lugol's iodine solution;
0.2 – 0.5 mL per 100 mL water sample.

Neutralised formaldehyde;
2 mL per 100 mL water sample.

The advantage of Lugol's iodine solution is that it has an instant effect and increases the weight of the organisms reducing sedimentation time. Lugol's iodine solution will cause discolouration of some phytoplankton making identification difficult. To reduce this effect, the sample can be bleached using sodium thiosulfate prior to analysis.

The advantage of formaldehyde is that preserved samples remain viable for a long time. Formaldehyde is not suitable for fixation of naked algal cells, as the cell shape is distorted and flagella are lost. Some naked algal forms may also disintegrate when formaldehyde is used (CEN 2005). Formaldehyde should be used with care because of its toxicity to humans (Andersen and Throndsen 2004).

Figure 5. Laboratory cell counter.

Figure 6. Computerised counting programme.

Storage of samples

Preserved phytoplankton samples should be stored in cool and dark conditions. When using Lugol's iodine solution, the colour of the sample should be checked regularly and if necessary, more preservative added. Preserved samples should be analysed without delay. Samples stored more than a year are of little use (Helcom Combine 2006).

Temperature adaptation

The first step in the analysis procedure is to adapt the phytoplankton sample and the sedimentation chamber to room temperature. This prevents convection currents and air bubbles forming in the sedimentation chamber. If this is not carried out non-random settling of the phytoplankton cells may occur.

Chamber preparation

Sediment chambers must be clean and dust free to avoid contamination from previous samples. Many laboratories use a new base plate after every sample. Sometimes it is necessary to grease the chimney bottom with a small amount of vaseline to ensure the chamber parts are tightly sealed (Andersen and Throndsen 2004).

In studies where the succession of the phytoplankton is examined over a period of time it is important to use the same chamber volume for the analysis (Hasle 1978a). At times, the "standard" chamber size may be either too small (extreme winter situations) or too large (phytoplankton blooms) and another chamber size must be used.

Sample homogenisation

Before the sample is poured into the sedimentation chamber, the bottle should be shaken firmly, but gently, in irregular jerks to homogenise the contents. Violent shaking will produce bubbles, which can be difficult to eliminate. A rule of thumb is to shake the bottle at least 50 times. It is recommended to check the homogenous distribution a couple of times per year by counting 3 subsamples from the same stock-sample.

Concentration/dilution of samples

Although it is possible to concentrate and dilute samples that are either too sparse or too dense it is not recommended as all additional handling steps may interfere with the sample contents. Instead it is recommended that a sediment chamber of an appropriate size be used to allow accurate identification and enumeration of cells.

Filling the sedimentation chamber

After homogenisation, the sedimentation chamber is placed on a horizontal surface and gently filled from the sample bottle (Fig. 7a and 7b). The chamber is then sealed with a cover glass. It is important that no air bubbles are left in the chamber. It may be necessary to grease the cover glass with a little vaseline to maintain a tight seal.

Sedimentation

The sedimentation should take place at room temperature and out of direct sunlight. In order to minimise evaporation the sedimentation chamber may be covered with a plastic box and a Petri dish containing water should be placed beside the chamber (Fig. 8). Settling time is dependent on the height of the chamber and the preservative used (Lund *et al.* 1958, Nauwerck 1963). Recommended settling times for Lugol's preserved samples are shown in Table 3. According to Hasle (1978a) formaldehyde preserved samples need a settling time of up to 40 hours independent of chamber size.

After sedimentation the chimney of the sedimentation chamber is gently slid off from the bottom plate and replaced by a cover glass. Care should be taken not to introduce airbubbles at this stage (Fig. 9). The transfer of the bottom plate to the microscope will not affect the distribution of the settled phytoplankton cells if there are no air bubbles present. The bottom plate is placed on the inverted microscope (Fig. 10) and the phytoplankton cells are identified and counted.

Table 3. Recommended settling times for Lugol's iodine preserved samples (from Edler 1979).

Chamber volume (mL)	Chamber height approx. (cm)	Settling time (hr)
2	1	3
10	2	8
25	5	16
50	10	24

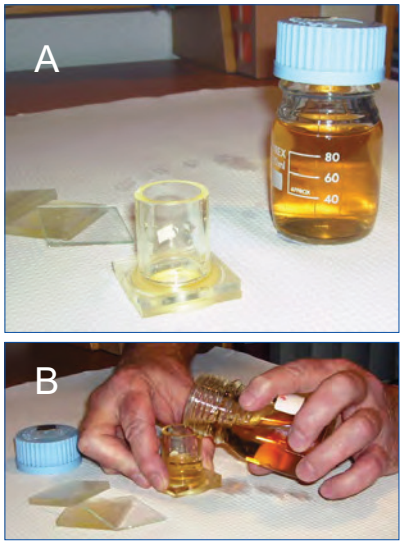

Figure 7A and 7B. Filling of sedimentation chamber.

Figure 8. Sedimentation, with a Petri dish filled with water. A plastic box covers the sedimentation chamber and the Petri dish to maintain the humidity.

Figure 9. Replacing the sedimentation chimney with a cover glass.

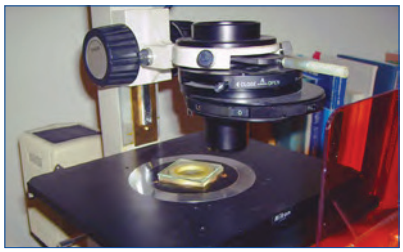

Figure 10. Chamber bottom placed in microscope ready for analysis.

Counting procedure

The quantitative analysis should start with a scan of the entire chamber bottom at a low magnification. This will help to give an overview of the density and distribution of phytoplankton. If the distribution is considered uneven the sample must be discarded. During this scan it is also convenient to make a preliminary species list, which may help to select the counting strategy.

Organisms should be identified to the lowest taxonomic level that time and skill permits (Hasle 1978b). Ultimately the objective of the study will decide the level of identification accuracy.

Counting begins at the lowest magnification, followed by analysis at successively higher magnification. For adequate comparison between samples, regions and seasons it is important to always count the specific species at the same magnification. In special situations, such as bloom conditions, however, this may not be possible. Large species which are easy to identify (e.g. *Ceratium* spp.) and also usually relatively sparse can be counted at the lowest magnification over the entire chamber bottom. Smaller species are counted at higher magnifications, and if needed, only on a part of the chamber bottom. In Table 4, the recommended magnifications for different phytoplankton sizes are listed.

Counting the whole chamber bottom is done by traversing back and forth across the chamber bottom. The parallel eyepiece threads delimit the transect where the phytoplankton are counted (Fig. 11).

Counting part of the chamber bottom can be done in different ways. If half the chamber bottom is to be analysed every second transect of the whole chamber is counted. If a smaller part is to be analysed one, two, three or more diameter transects are counted. After each transect is counted the chamber is rotated 25-45° (Fig. 12).

When counting sections of the chamber using transects it is important to be consistent as to which cells lying on the border lines are to be counted. The easiest way is to decide that cells lying on the upper or right line should be counted, whereas cells on the lower or left line should be omitted.

In order to obtain a statistically robust result from the quantitative analysis it is necessary to count a certain number of counting units (cells, colonies or filaments). The precision

Table 4. Recommended magnification for counting of different size classes of phytoplankton (Edler, 1979, Andersen and Throndsen 2004).

Size class	Magnification
0.2 – 2.0 µm (picoplankton)*	1000 x
2.0 – 20.0 µm (nanoplankton)	100 – 400 x
>20.0 µm (microplankton)	100 x

* picoplankton are normally not analysed using the Utermöhl method.

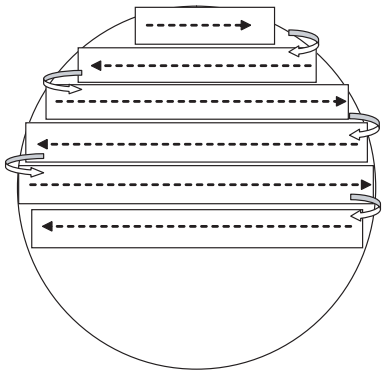

Figure 11. Counting of the whole chamber bottom with the parallel eyepiece threads indicating the counted area.

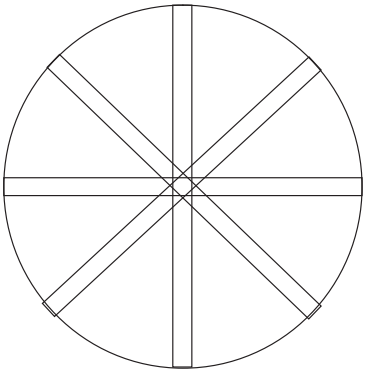

Figure 12. Counting of diameter transects.

Table 5. Relationship between number of cells counted and confidence limit at 95% significance level (Edler 1979, Andersen and Throndsen 2004).

No of counted cells	Confidence limit +/- (%)	Absolute limit if cell density is estimated at 500 cells L^{-1}
1	200	500 ± 1000
2	141	500 ± 705
3	116	500 ± 580
4	100	500 ± 500
5	89	500 ± 445
6	82	500 ± 410
7	76	500 ± 380
8	71	500 ± 355
9	67	500 ± 335
10	63	500 ± 315
15	52	500 ± 260
20	45	500 ± 225
25	40	500 ± 200
50	28	500 ± 140
100	20	500 ± 100
200	14	500 ± 70
400	10	500 ± 50
500	9	500 ± 45
1000	6	500 ± 30

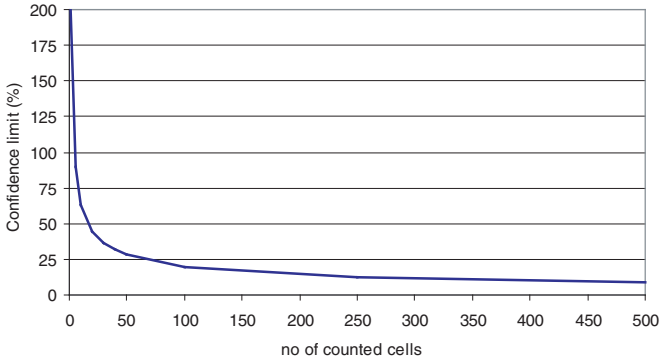

Figure 13. Relationship between number of cells counted and confidence limit at the 95% significance level.

desired decides how many units to count. The precision is usually expressed as the 95% confidence limit as a proportion of the mean. Table 5 and Figure 13 show the relationship between number of units counted and the accuracy. In many studies it has been decided that counting of 50 units of the dominant species, giving a 95% confidence limit of 28% is sufficient. Increasing the precision to e.g. 20% or 10% would need a dramatic increase in counted units, 100 and 400 respectively (Venrick 1978, Edler 1979). The precision is given by the following equation:

$$Precision\,\% = \frac{2*100}{\sqrt{number\ of\ cells\ counted}}$$

It is clear that it will not be possible to count 50 units of all species present in a sample. Some species may not be sufficently abundant which will decrease the overall precision. To maintain an acceptable precision for the entire sample a total of at least 500 units should be counted (Edler 1979).

The counting unit of most phytoplankton species is the cell. In some cases this is not practical. For filamentous cyanobacteria, for instance, the practical counting unit is a certain length of the filament, usually 100 µm (Helcom Combine 2006). In some colony forming species and coenobia it may be difficult to count the individual cells. In such cases the colony/coenobium should be the counting unit. If desired, the calculation of cells per colony/coenobium can be approximated by a thorough counting and mean calculation of a certain number of colonies/coenobia.

The transformation of the microscopic counts to the concentration or density of phytoplankton of a desired water volume (usually Litre or millilitre) can be achieved using this equation:

$$Cells\,L^{-1} = N * \left(\frac{A_t}{A_c}\right) * \frac{1000}{V}$$

$$Cells\,mL^{-1} = N * \left(\frac{A_t}{A_c}\right) * \frac{1}{V}$$

V: volume of counting chamber (mL)
A_t: total area of the counting chamber (mm^2)
A_c: counted area of the counting chamber (mm^2)
N: number of units (cells) of specific species counted
C: concentration (density) of the specific species

Cleaning of sedimentation chambers

The cleaning of sedimentation chambers is a critical part of the Utermöhl method. The chambers should be cleaned immediately after analysis to prevent salt precipitate formation. A soft brush and general purpose detergent should be used (Edler 1979, Tikkanen and Willén 1992). To clean the chamber margin properly a tooth pick can be used. Usually it is sufficient to clean the chamber bottom without dissembling the bottom glass. Sometimes, however, it is necessary to separate the bottom glass from the chamber, either to clean it or to replace it. This is easily done by loosening the ring holding the bottom glass with the key. Care should be taken as the bottom glasses are very delicate. Counting chambers should be checked regularly to ensure that no organisms stick to the bottom glass. This can be achieved by filling the chambers with distilled water.

Quality assurance

To ensure high quality results all steps of the method must be validated. Ideally this is performed on natural samples, but in some instances it may be helpful to spike the sample with cultured algae. Steps in the Utermöhl method to validate are

- homogenisation of sample
- sedimentation/sinking
- distribution on chamber bottom
- repeatability and reproducibility

Ultimatley the quality of the result from this method is dependent on the skill of the analyst. The variation of parallel samples counted by the same analyst and the variation in parallel samples counted by different analysts are two of the most important considerations in quality assurance (Willén 1976). When possible laboratories should take part in interlaboratory comparisons.

Epifluorescence microscopy

Epifluorescence microscopy is an effective method to enhance detection and identification of certain organisms (Fritz and Triemer 1985, Elbrächter 1994). In formalin fixed samples, autofluorescence of the chlorophyll can easily be detected by epifluorescence. This will be specially important among dinoflagellates and euglenids, in which both phototrophic and obligate heterotrophic genera/species are present. Phycobilins of cyanobacteria, rhodophytes and cryptophytes have a special autofluorescence, thus this method is particularly suited to detect and count cryptophytes and small coccoid cyanobacteria. In addition, staining of organisms can help to enhance counting effort and identification of certain organisms. Applying this method, the inverted microscope should have an epifluorescence equipment. The lenses should be suitable for fluorescence microscopy. For the respective excitation filter and barrier filter to be used to detect the different epifluorescence emissions, the supplier of the respective microscope should be contacted. Some information on filter combinations is provided by Elbrächter (1994). A common method is to induce epifluorescence in organisms with cellulose cell walls (e.g. thecate dinoflagellates, chlorophytes, “fungi” and others) by Fluorescent Brightener (Fritz and Triemer 1985).

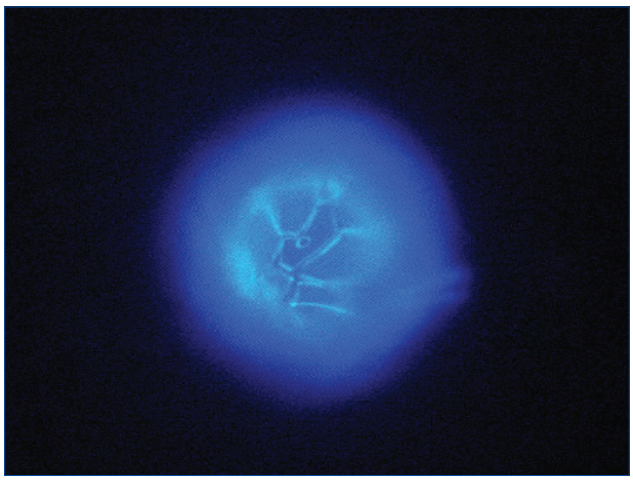

Figure 14. *Alexandrium ostenfeldii*, epifluorescence light microscopy, stained with Fluorescent Brightener. Note the clear indication of the sutures and the large ventral pore, characteristic for this species.

Protocol for staining and use of epifluorescence

- Prepare a 0.1% stock solution of Fluorescent Brightener.
- The fluorescent brightener solution should be added to the sedimentation chamber before filling it with the sample. The final concentration should be 0.02 %.
- Switch on the mercury lamp for about 10 min. before starting to analyse the sample.
- Use Exitation Filter BP 390-490 and Barrier Filter LP 515 or filters recommended by the microscope brand.

This will give dinoflagellate thecae a clear intensive blue epifluorescence including the sutures of the plates (Fig. 14). Other cellulose items like chlorophyte cell walls, cell walls of fungi parasitising in diatoms etc. will also fluoresce.
Note that the intensity of epifluorescence is pH dependent, in acidic samples epifluorescence is absent or poor.

Discussion

The Utermöhl method for the examination of phytoplankton communities is probably the most widely used method for the quantitative analysis of phytoplankton. Through the years both microscopes and sedimentation chambers have developed considerably, yet it is the taxonomic skill of the analyst that sets the standard of the results.

The Utermöhl method determines both the quantity and diversity of phytoplankton in water samples. Moreover, with only a little extra effort, the biovolume of the different species can also be elucidated. The method allows very detailed analysis and with high quality lenses the resolution of phytoplankton morphology can be very good. The Utermöhl method has some disadvantages. It is very time consuming and thus also very costly. In order to achieve reliable results the analyst has to be skilled, with a good knowledge of the taxonomic literature. It is commonly agreed that analysts take some years to train and must then keep up to date with the literature.

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