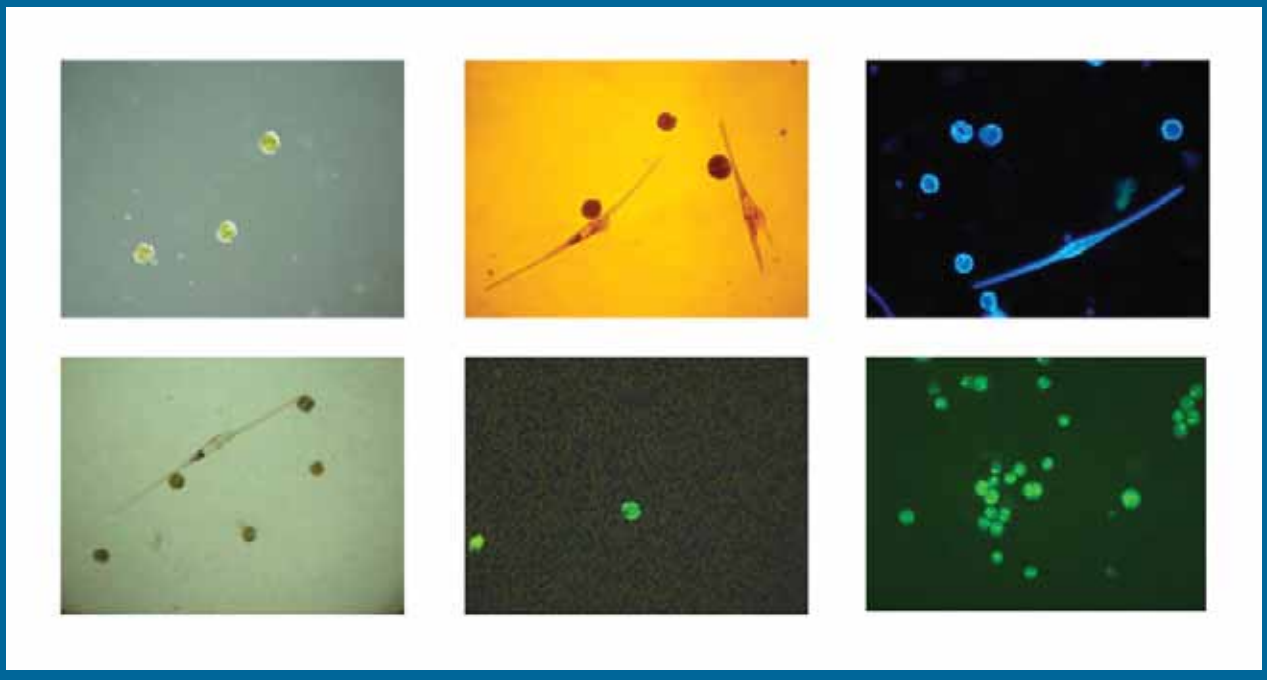
Intergovernmental Oceanographic Commission

Manuals and Guides 55

MICROSCOPIC AND MOLECULAR METHODS FOR QUANTITATIVE PHYTOPLANKTON ANALYSIS



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No.	Title
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2	International Catalogue of Ocean Data Station. 1976. (Out of stock)
3 rev. 3	Guide to Operational Procedures for the Collection and Exchange of JCOMM Oceanographic Data. Third Revised Edition, 1999. 38 pp. (English, French, Spanish, Russian)
4	Guide to Oceanographic and Marine Meteorological Instruments and Observing Practices. 1975. 54 pp. (English)
5 rev. 2	Guide for Establishing a National Oceanographic Data Centre. Second Revised Edition, 2008. 27 pp. (English) (Electronic only)
6 rev.	Wave Reporting Procedures for Tide Observers in the Tsunami Warning System. 1968. 30 pp. (English)
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	Vol. 6: Quick Reference Sheets for GF3 and GF3-Proc. 1989. 22 pp. (English, French, Spanish, Russian)
18	User Guide for the Exchange of Measured Wave Data. 1987. 81 pp. (English, French, Spanish, Russian)

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MICROSCOPIC AND MOLECULAR METHODS FOR QUANTITATIVE PHYTOPLANKTON ANALYSIS

editors

Bengt Karlson, Caroline Cusack and Eileen Bresnan

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Preamble

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The Intergovernmental Oceanographic Commission of UNESCO has since 1992 given attention to activities aimed at developing capacity in research and management of harmful microalgae. With this IOC Manual & Guide we wish to fill a gap for information and guidance, in an easy accessible and low cost format, to comparison between traditional and modern methods for enumeration of phytoplankton. Enumeration of harmful phytoplankton species is a key element in many monitoring programmes to protect public health, seafood safety, markets, tourism, etc. However, phytoplankton enumeration has self evidently much broader application that just monitoring of harmful microalgae species.

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 facilitate further research and especially systematic observations and data gathering. The results include the publications in the 'IOC Manuals and Guides' series, and the UNESCO series 'Monographs in 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 in the framework of the Global Ocean Observing System.

The IOC is highly appreciative of the efforts of the ICES-IOC Working Group on Harmful Algal Bloom Dynamics in organizing the Joint ICES-IOC Intercomparison Workshop on New and Classic Techniques for Estimation of Phytoplankton Abundance at the Kristineberg Marine Research Station in Sweden 2005, and not the least the efforts of the scientists who prepared the manuscripts for this IOC Manual & Guide. The IOC wishes to express its particular thanks to Dr. Bengt Karlson, SMHI Sweden, Editor-in-Chief, for his determination to produce this volume.

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

The publication of this IOC Manual & Guide has been made possible through support from the United States National Oceanic and Atmospheric Administration and the Department of Biology, University of Copenhagen, Denmark.

Henrik Enevoldsen

IOC Harmful Algal Bloom Programme
http://ioc.unesco.org/hab

Foreword

Phytoplankton occupy the base of the food web of the sea. It plays a vital role in the global carbon cycle and is also of importance since some phytoplankton may cause harmful algal blooms, a problem e.g. for aquaculture. Man induced changes in the environment, e.g. eutrophication, can be manifested in changes in the phytoplankton community and there is now some evidence that climate change may also be having an effect. Phytoplankton analysis is an essential part in the process of understanding and predicting changes in our environment. Recent introduction of new methods, several based on molecular biology, has led to a perceived need for a manual on quantitative phytoplankton analysis.

The aim of this publication is to provide a guide for phytoplankton analysis methods. A number of different methods are described and information about applicability, cost, training, equipment etc. is included to facilitate information on choosing the right method for a certain purpose. The costs of equipment, consumables, etc. are based on 2009 prices. Although the methods described are for marine plankton they are also applicable for freshwater plankton. The method descriptions are more detailed than what is usually found in scientific articles to make the descriptions useful when setting up monitoring or research programmes that include inexperienced researchers. Some of the methods described are relatively old and well tested while a few must be considered to be emerging technology. We hope that this publication will supplement existing literature and that the distribution of the book freely using the Internet will make it useful in environmental monitoring and for students, researchers and regulators. A book like this can never be complete. Some methods are missing and newer techniques are under development.

The production of this book was initiated during an international workshop at Kristineberg Marine Research Station in Sweden 2005. Participants in the *Joint ICES/IOC Intercomparison Workshop on New and Classic Techniques for Estimation of Phytoplankton Abundance (WKNCT)* agreed to write chapters of the book. A scientific paper describing the results of this workshop can be found in Godhe *et al.* (2007). Co-authors have joined some of the workshop participants. The Harmful Algal Bloom programme of the Intergovernmental Oceanographic Commission, of UNESCO, has aided in the production and also financed the printing of the book. We would like to express our gratitude to everyone who has been involved in the production of this book. In particular the editors would like to acknowledge the time and effort contributed to the final edits and proof reading by Jacob Larsen and Pia Haecky.

Bengt Karlson, Caroline Cusack and Eileen Bresnan

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Godhe A, Cusack C, Pedersen J, Andersen P, Anderson DM, Bresnan E, Cembella A, Dahl E, Diercks S, Elbrächter M, Edler L, Galuzzi L, Gescher C, Gladstone M, Karlson B, Kulis D, LeGresley M, Lindahl O, Marin R, McDermott G, Medlin MK, Naustvoll L-J, Penna A, Töbe K (2007) Intercalibration of classical and molecular techniques for identification of *Alexandrium fundyense* (Dinophyceae) and estimation of cell densities. Harmful Algae 6: 56-72

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).

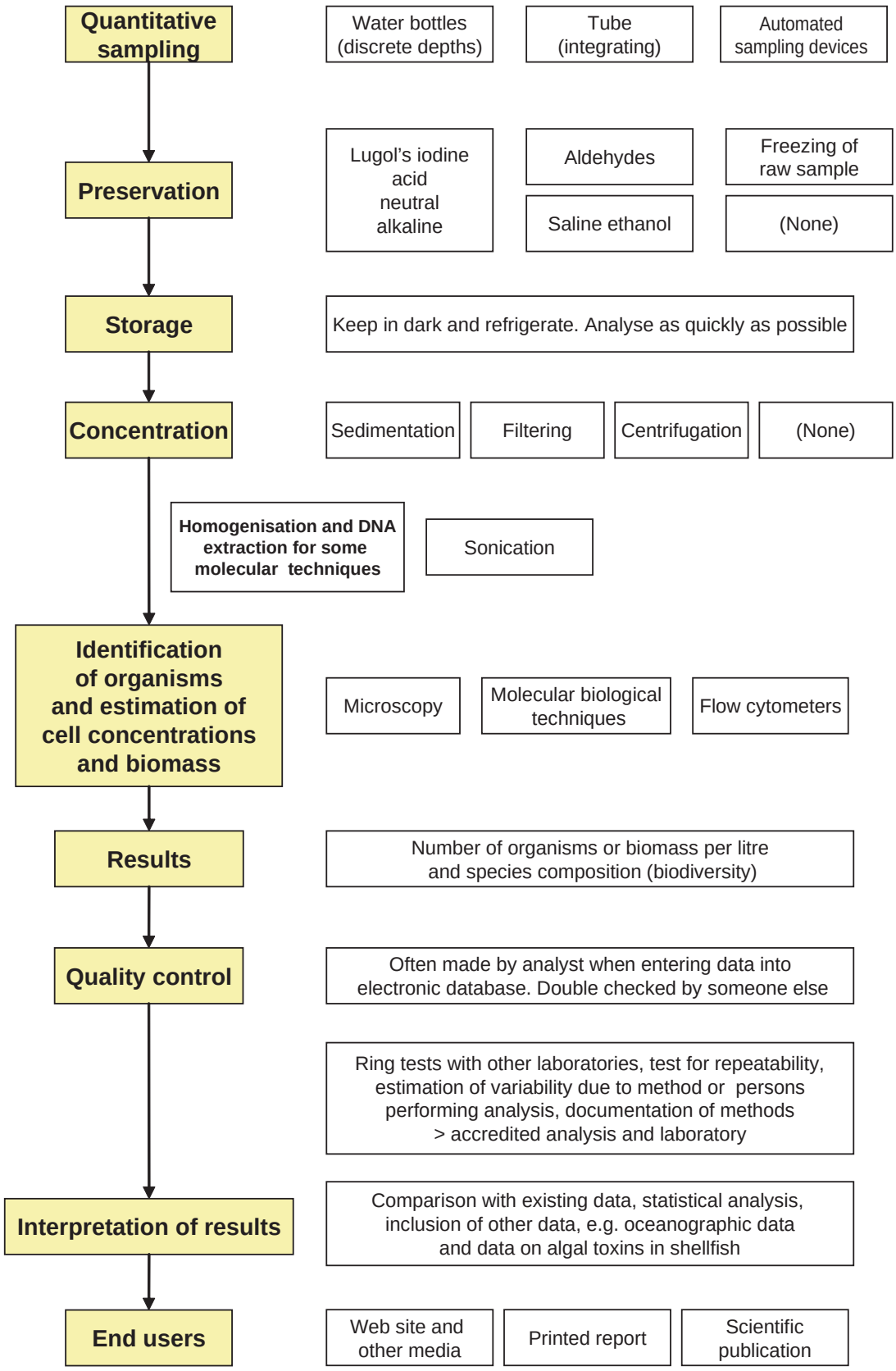

Figure 1. Schematic drawing of the steps from sampling to results.

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

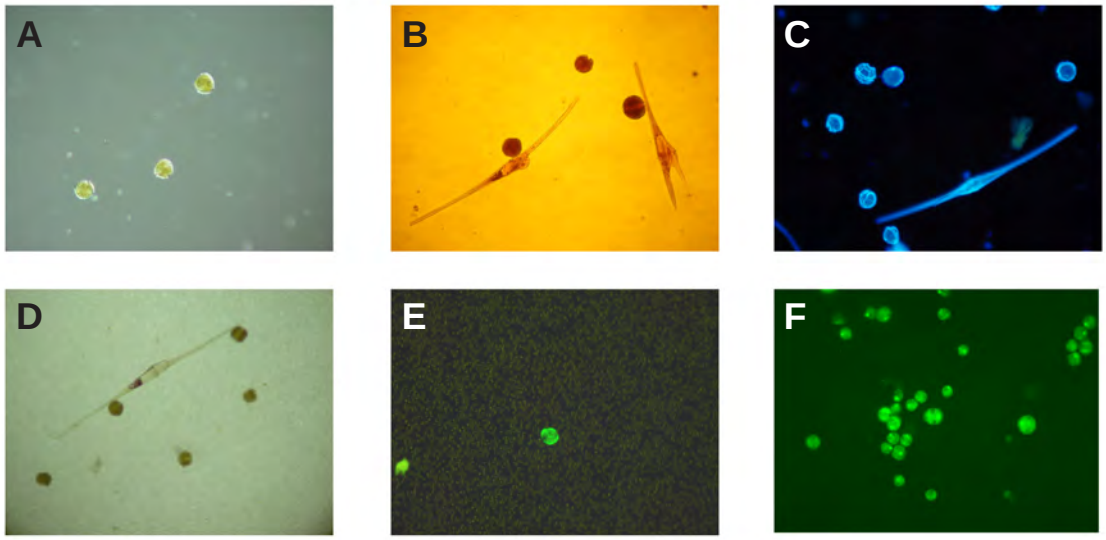

Figure 10. *Alexandrium fundyense* as seen in the microscope using different techniques. *Ceratium* spp. are present in B, C and D. A. Filter freeze transfer with contrast enhancement using DIC (appearance in Utermöhl is essentially identical), B. Sedimentation flask, C. Filtering + calcofluor staining, 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.

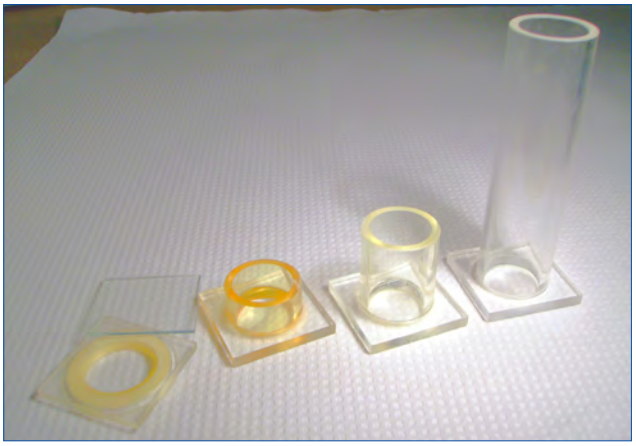

Figure 2. Sedimentation chambers. From left to right: bottom plate with cover glass, 10 mL chamber, 25 mL chamber and 50 mL chamber.

Figure 3. Inverted microscope.

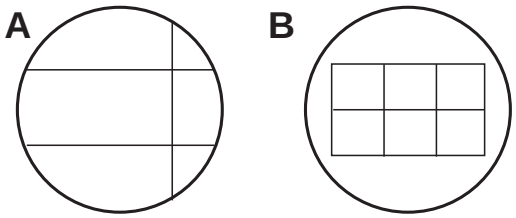

Figure 4. Counting aids mounted in the eyepiece. a) parallel threads, with a transverse thread. b) grids.

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

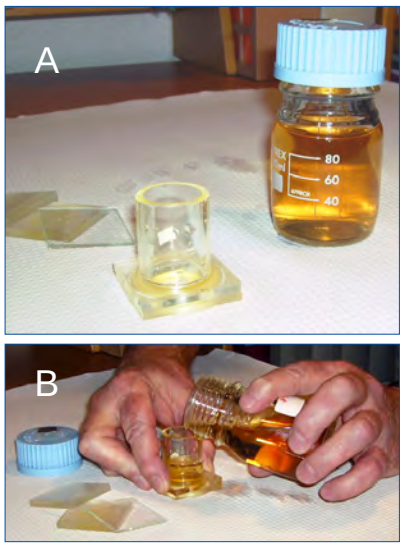

Figure 7A and 7B. Filling of sedimentation chamber.

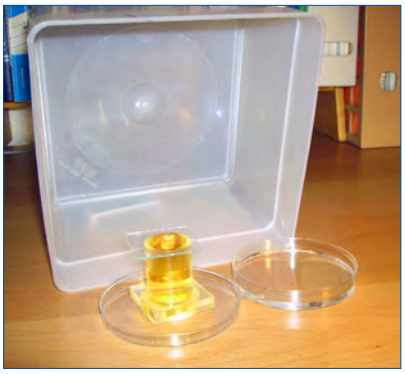

Figure 8. Sedimentation, with a Petri dish filled with water. A plastic box covers the sedimentation chamber and the Petri dish to maintain the humidity.

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

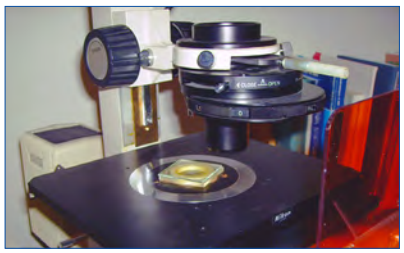

Figure 10. Chamber bottom placed in microscope ready for analysis.

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.

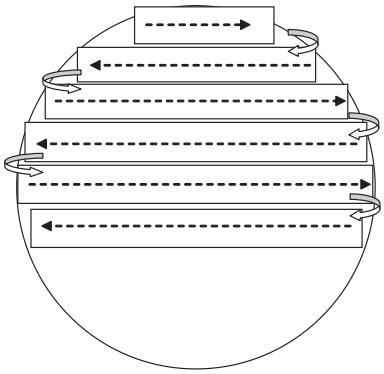

Figure 11. Counting of the whole chamber bottom with the parallel eyepiece threads indicating the counted area.

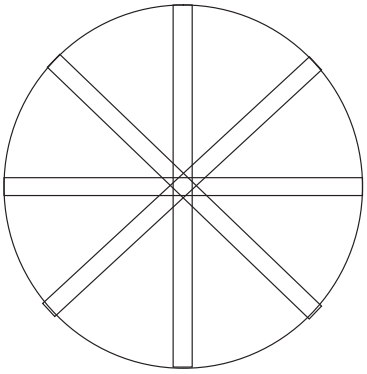

Figure 12. Counting of diameter transects.

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

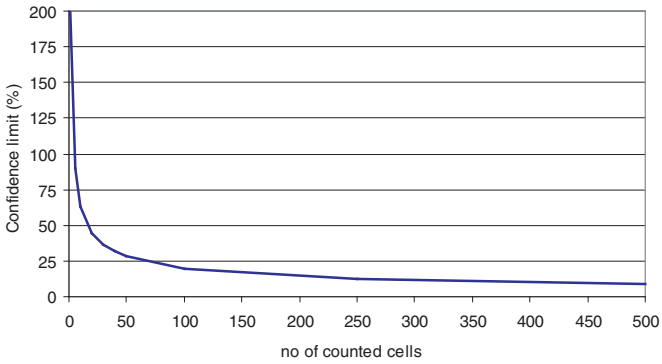

Figure 13. Relationship between number of cells counted and confidence limit at the 95% significance level.

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).

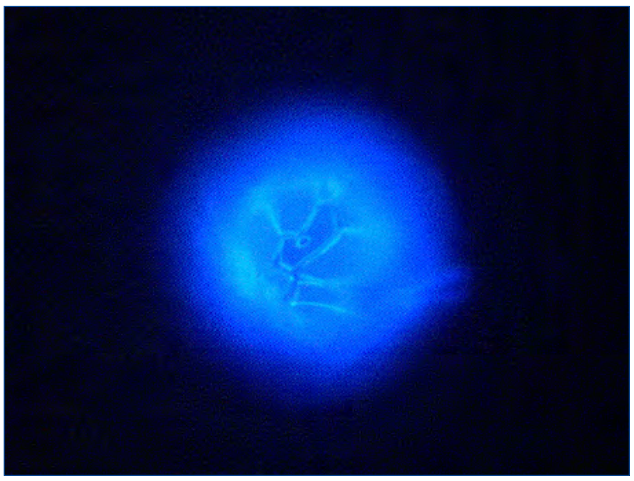

Figure 14. *Alexandrium ostenfeldii*, epifluorescence light microscopy, stained with Fluorescent Brightener. Note the clear indication of the sutures and the large ventral pore, characteristic for this species.

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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3 Settlement bottle method for quantitative phytoplankton analysis

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Introduction

The settlement bottle technique is a modified Utermöhl technique (Hasle 1978) for quantifying phytoplankton. It relies on the observation and enumeration of phytoplankton cells after sedimentation using an inverted microscope. It differs from other similar methodologies as once a sample has been taken and is preserved, there is no further requirement for any more sub-sampling or manipulation. Once a water sample has been taken, it is transferred directly into a plastic tissue culture flask, a preservative is added and the sample is stored until analysis. For counting, the contents of the flask are gently shaken, allowed to settle onto one of the flat sides of the flask which is then placed directly onto an inverted microscope. As with other Utermöhl methods, it requires the skills of an analyst experienced in the identification of phytoplankton cells.

Materials

Laboratory facilities

The settlement bottle method is simple in that all it requires are tissue culture flasks (acting as sample jars and counting chambers), some preservative and an inverted microscope. Thus all that is required is a room, preferably without direct sunlight, with enough space and a power supply to use an inverted microscope.

Equipment

Tissue culture bottles of 50-60 mL capacity are used. Larger volume bottles are difficult to place on a microscope and suffer from movement of water inside them during examination. Smaller sized bottles will probably not contain enough sample. The bottles should be rectangular in shape, as opposed to having a triangular form, as this makes the calculation at the end much simpler.

The base of the tissue culture bottles are examined using an inverted microscope. This should be equipped with 10X, 20X and 40X objective lenses. The 20X and 40X lenses should have a long focal length. The technique can be applied using a choice of either brightfield or phase contract microscopy. Differential interference contrast (DIC) and epifluorescence microscopy are unsuitable. A specialised plate for secure mounting of the settlement bottle onto the stage of the inverted microscope may need to be manufactured. See Appendix for examples of equipment.

Chemicals and consumables

The only chemicals that are required are solutions of preservative. Lugol's iodine, neutral formalin or glutaraldehyde are all suitable.

Methods

Preparation of sample

Before taking a sample, a tissue culture bottle should be labelled with appropriate information (Date, location, station number, depth) with a permanent marker pen. Labels should be written on the edge or narrow side of the bottle, not on the broad side (see Fig. 1) so that it does not interfere with the identification and enumeration of cells.

The tissue culture bottle should be filled to the top with the water sample, leaving just enough air space to add preservative. This prevents the introduction of large air bubbles which can degrade the optical path when examining through or near the edge of the bubble.

Before the sample is allowed to settle it should be wiped clean and acclimated to room temperature for 24 hours and then gently shaken to disrupt any aggregation of phytoplankton cells. This can be achieved by a combination of horizontally rolling and vertically turning the sample bottle upside down as gently as possible to prevent the break-up of colonies and the accumulation of air bubbles. The contents of the tissue culture flask should be allowed to settle for a period of at least six hours.

Analysis of sample

The phytoplankton cells can then be counted using an inverted microscope. The entire base of the bottle or a number of strips, going across the length or width of the bottle, can be examined and the cells of each species or type are scored (see Fig. 2 for details).

Preservation and storage

As with all samples of phytoplankton, deterioration is extremely fast in direct sunlight. All samples should therefore be stored in the dark, stacked, preferably horizontally, to minimise storage space. Samples should also be kept cool, although refrigeration is not absolutely necessary. Storage over long periods of time (months) is not as effective using Lugol's iodine, as opposed to formalin or glutaraldehyde, as leaching of iodine into the plastic will occur which will deteriorate the quality of microscopic observations.

The fundamentals of

The settlement bottle method

Scope
Qualitative and quantitative analysis of phytoplankton.

Detection range
Detection range is dependent on the volume of sample settled and the number of strips analysed. Counting over the base of a 50 mL settlement bottle will give a detection limit of ca. 20 cells per Litre.

Advantages
Avoidance of errors arising from sub sampling. Qualitative as well as quantitative analysis. Identification and quantification of multiple or single species. Detection of harmful species. Sediment bottles are available at low cost.

Drawbacks
Optical resolution is reduced due to the thickness of the wall of the settlement bottles. This is a time consuming analysis that requires skilled personnel. Sedimentation time prevents the immediate analysis of samples. DIC and epifluorescence microscopy are not suitable with this method. Special long distance objectives must be used at higher magnifications.

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

Essential Equipment
Inverted microscope, settlement bottles, identification literature.

Equipment cost*
Inverted microscope: 7,500 – 50,000 € (11,000 – 70,000 US $).
Identification literature: 1,000 – 3,000 € (1,400 – 4,300 US $).

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

Processing time per sample before analysis
A minimum of 6 hours sedimentation time for a 50 mL settlement bottle.

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

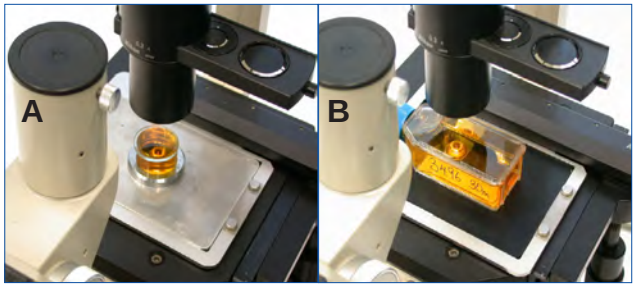

Figure 1. A comparison of a) the Utermöhl settlement chamber and b) the settlement bottle method for counting phytoplankton cells using an inverted microscope. In the example above a 10 mL settlement chamber shows a similar height of water from which samples are sedimented as the 50 mL cell culture bottle used in b), allowing the same density of cells on the base of each. However, a much bigger surface area can be examined when using the settlement bottle, allowing more accurate estimations of, in particular, the larger species since more cells are counted. This can be done quite rapidly at lower power magnifications, for example using a X10 objective lens. Note the sample label of station number and depth is written on the side of the bottle (date and location are on the opposite side). Note also that special mounts on the movable stage are required for both types of settlement container.

Calculation of results

Once a count of a particular species or type has been completed, this must be multiplied by a conversion factor (in this case *F*) to calculate the cell density per unit volume. This type of conversion applies to all sedimentation techniques for enumerating phytoplankton cells. The general formula for achieving a value of *F* to derive the cell density in cells per Litre is:

$$F = \frac{\text{Settling area}}{\text{Area analysed}} * \frac{1000\ (\text{mL})}{\textit{Sample volume}}$$

The value of *F* will vary when counting in strips along the bottle. This will depend on the magnification of the objective lens, the number of strips across the bottle which were examined, and whether the strips were oriented along or across the bottle. The width of the field of view (i.e. the width of each strip) must be predetermined using a calibrated graticule at each magnification. If one is scanning the long side of the bottle, the length of the short side across the bottle is required, and vice versa.

A worked example

A tissue culture bottle of 50 mL capacity was filled with a sea water sample and preserved with Lugol's iodine. The content of the bottle was gently shaken and allowed to settle onto the flat side of the bottle; dimensions 60 x 30 mm. Five strips across the long length of this flat side were examined using a 20X objective lens. The width of the field of view was previously estimated as 0.95 mm at this magnification. A total of 135 cells of *Karenia mikimotoi* were counted.

In this example five strips across the long side were originally examined (as shown in Fig. 2b) the value for F then becomes:

$$F = \frac{30}{5*0.95} * \frac{1000}{50} = 126.3$$

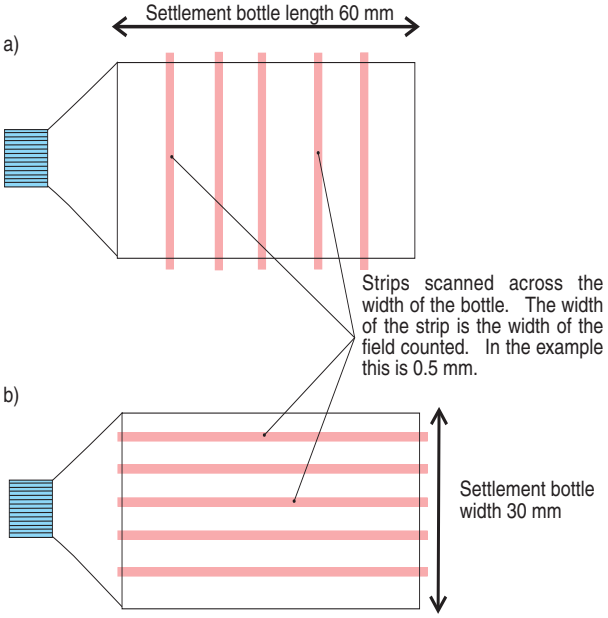

Figure 2. Methods of counting strips (shaded areas) across a cell culture bottle. If, for example, the width of the field of view, and hence of each strip is 0.95 mm, then the *F* factor may be calculated. In a) where the strips are widthways the ratio of total area to the area counted is 60/(5 * 0.95). In b) with the strips lengthways this ratio is 30/(5 * 0.5). With a sample volume inside the bottle of 50 mL, the *F* factor for a) then becomes (60/(5 * 0.95)) * (1000/50) = 12.6 * 20 = 252. In b) the *F* factor is (30/(5*0.95))*(1000/50) = 6.3 * 20 = 126. *F* factors can become very high using the cell culture bottle technique unless a suitable number of strips are counted.

Where 30 equals the width of the bottle (mm)
5 equals the number of strips counted
0.95 equals the width of the transect counted (mm)

The density of *K. mikimotoi* cells in the sample then becomes:

135 * 126.3 = 17,000 cells per Litre.

The standard error (SE) using this technique, as a percentage, is typically the square root of the number of cells counted, expressed as a proportion of the cells counted:

$$\frac{\sqrt{135}}{135} = \frac{11.6}{135} = 8.6\%$$

This gives an overall result of a cell density for *K. mikimotoi* of 17,000 cells per Litre ± 1,500. Note the number of significant figures in this result (1,500 has been rounded from 1,467).

Discussion

A common problem in plankton identification and enumeration is archiving a particular sample for future reference, verification of the identity of a species or even accurate intercalibration studies. A particular advantage of the settlement bottle technique is the minimisation of sample handling which can, at times, introduce serious errors. A sample is di-

rectly put into a tissue culture bottle, preserved and capped in the field, after which there is no further need for any future sub-sampling. Samples fixed with formalin can be stored intact for an extended period of time. If noxious chemicals such as formalin or glutaraldehyde are used as preservatives, then there is no danger of contamination or fumes resulting from them as the sample is in an air- and water-tight container. Additionally, many samples can be prepared for analysis at any given time as there is no need for an extended range of relatively expensive settlement chambers.

No technique is without imperfections. In the case of the settlement bottle method the most irritating of these is probably the leaching of iodine into the sides of the tissue culture bottle if either acidic or neutral Lugol's iodine is used to preserve the samples. This occurs after a few weeks, and reduces the quality of the observations. As the sample is enclosed, sedimented specimens of phytoplankton cannot be manipulated which can on occasion make identification very difficult. Observations of cells at the extreme edges sides of the bottle can be difficult. However, the method is ideal for long-term storage especially if the samples are stored with formalin. The method is also very low cost relative to other techniques.

Acknowledgements
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Appendix

Table 1. Equipment and suppliers.

Equipment	Supplier and model reference	€	US $
Inverted Microscope	e.g. Nikon TS100F, Olympus CKX41	5,000-6000†	6,500-8,500
Tissue Culture Bottles	Any medical laboratory supplier	0.5-1 each	Ca. 1 each

†The price can vary considerably depending on the quality of the objective lenses used. The price here is for a reasonable quality set of 10X, 20X and 40X lenses.

4 Counting chamber methods for quantitative phytoplankton analysis - haemocytometer, Palmer-Maloney cell and Sedgewick-Rafter cell

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General introduction

The counting chamber methods are established methods to count phytoplankton. The three most common types of counting chambers for phytoplankton enumeration are the Sedgewick-Rafter counting slide, the Palmer-Maloney counting slide and the haemocytometer counting slide. All three methods are easy to learn and use, requiring preserved sample, a good quality compound microscope and counting slides. The set up cost is low and it is beneficial to have a selection of all three on hand in the laboratory. These methods are particularly suitable for samples containing a high concentration of cells, as in bloom situations or in phytoplankton cultures with the haemocytometer being reserved for extremely high cell densities of small organisms. The capability of the Sedgewick-Rafter and the Palmer-Maloney to view the whole phytoplankton community including the presence of harmful species is a definite asset. Nevertheless correct identification of the phytoplankton community still requires highly trained analysts for its implementation.

Sedgewick-Rafter counting slide

Introduction

The Sedgewick-Rafter counting slide is a traditional counting method using a compound microscope and a highly trained taxonomist. This is a rapid method for quantifying samples with high cell numbers. The slide is comprised of a transparent base, which has a centrally mounted chamber (50 mm x 20 mm x 1 mm deep) and can hold 1 mL of sample. The base of this chamber has a ruled 1 mm grid, so that the 1 mL sample is subdivided into single microlitres. This chamber is covered over by a cover glass, which protects the sample from drying out and disturbances by air currents. The sample is then counted using a compound microscope.

Materials

Equipment

- A standard, compound microscope with 10X and 20X objectives and brightfield and phase contrast. A 40X objective may not be able to be used for this analysis, this depends on the working distance of the objective lenses.
- A Sedgewick-Rafter slide: This can be made from plastic or glass.
- A tally counter is useful when counting high cell numbers.

Chemicals and consumables

- A pipette is needed to dispense the sample into the cell.
- Sample bottles for storing the samples.

Solutions for preservation

- Lugol's iodine or formalin.

Methods

1 Prior to analysis the sample should first be homogenised. This is achieved using a combination of horizontally rolling and vertically turning the sample bottle as gently as possible to prevent the breakup of colonies and the accumulation of bubbles;

2 From a well mixed sample, 1 mL is removed using a pipette. The pipette should have a wide opening that does not restrict the movement of larger phytoplankton species (such as *Noctiluca scintillans*, *Ceratium* species). This is especially important when using a 1 mL pipette with removable tips, the end of the tip should be cut off to widen the opening;

3 The cover glass should be placed carefully onto the counting slide, perpendicular to the long axis of the slide, so one corner is left open for filling and another for the escape of air;

4 The sample aliquot is then dispensed into the counting cell (Fig. 1a – f):

5 Slowly swing the cover glass so that it completely covers the sample. Careful alignment of the cover glass will prevent air bubbles from being introduced into the sample and will ensure that the sample holds its complete volume. If a bubble develops, refill the counting cell.

6 Preserved samples should be left to settle for 15 minutes before enumeration;

7 The sample is then examined using a compound microscope. The slide should first be scanned under low magnification to estimate the concentration of cells. Using this information a counting strategy is decided upon as to whether the whole slide or a noted fraction is to be counted;

8 If the concentration of phytoplankton in the Sedgewick-Rafter slide is too dense and the cells are overlapping thus hindering identification, a dilution step should be performed using filtered seawater for marine samples;

9 After analysis is completed the slide is washed and cleaned between samples to prevent cross-contamination. A pure detergent like soap is recommended (Hallegraeff *et al.* 2004);

The fundamentals of

The counting chamber methods

Counting chamber	Sedgewick-Rafter	Palmer-Maloney	Haemocytometer
Scope	Cultures and high cell numbers	Cultures and high cell densities as in bloom conditions	Cultures and extremely high cell concentration of small organisms
Detection range	1,000 cells L^{-1} Limit of Detection (LOD)	10,000 cells L^{-1} (LOD)	10,000,000 cells L^{-1} (LOD)
Advantages	A rapid estimate of cell concentrations	A rapid estimate of high cell concentrations	A rapid estimate of extremely high cell concentrations
Drawbacks	Accurate results only when sample contains high phytoplankton cell densities	Accurate results only when sample contains **very** high cell densities	Accurate results only when sample contains **extremely** high cell densities
Type of training needed	Method-easy to learn and use. Highly trained taxonomist needed for verification of species identification	Method-easy to learn and use. Highly trained taxonomist needed for verification of species identification.	Method-easy to learn and use. Highly trained taxonomist needed for verification of species identification
Essential Equipment	Compound Microscope Cover slips Pipettes Sedgewick-Rafter slides	Compound Microscope Cover slips Pipettes Palmer Maloney slides	Compound Microscope Pipettes Haemocytometer slide with cover glass
Equipment cost	Compound Microscope: € 2500 / 3250 US $ Sedgewick-Rafter slides: Perspex :€50/ 65 US $ Glass: €166/ 213 US $	Compound Microscope: € 2500 / 3250 US $ Palmer-Maloney slides: Ceramic- €60/ 80 US $ Stainless Steel-€170/$230 US $	Compound Microscope: € 2500 / 3250 US $ Haemocytometer slide: €200/ 230 US $
Consumables, cost per sample	€ 1/$1.3 US $	€ 1/$1.3 US $	€ 1/$1.3 US $
Processing time/sample:	20 minutes	5 minutes	5 minutes
Analysis time /sample:	This depends on the sample density	10-30 min/ sample depending on the sample density	< 20 min / sample depending on the sample density
Sample/throughput/person/ day	This depends on the sample density	14-20 dependent on target species and density of samples	< 30 dependent on target species
Samples processed in parallel	Only one sample at a time	Only one sample at a time	Only one sample at a time
Health and Safety issues	Dependent on preservative used	Dependent on preservative used	Dependent on preservative used

Preservation and storage

Samples should be stored in the dark (closed boxes) to avoid direct light (which affects the Lugol's iodine preservative) and at room temperature. Samples preserved with Lugol's iodine can deteriorate over time resulting in a paler solution which could impare preservation. If the samples are stored for extended periods, preservation should be checked regularly and further Lugol's iodine added as appropriate.

Formulas for calculating results

The Sedgewick-Rafter slide has a volume of 1 mL with the base of the cell being divided into 1,000 squares (50 rows by 20 rows), each representing 1/1,000 of the volume of the slide.

$$F = \frac{1{,}000}{Number\ of\ Squares\ \ counted} * 1{,}000$$

To obtain a final result expressed as cells L^{-1}, the following equation is used to calculate the multiplication factor (*F*). *F* is dependent on the number of squares of the base of the cell counted during the analysis.
Examples of *F* for the Sedgewick-Rafter slide:

4 rows (200 squares) are counted.

$$F = \frac{1{,}000}{200} * 1{,}000 = 5 * 1{,}000 = 5{,}000$$

50 rows (1000 squares) or the entire slide is counted.

$$F = \frac{1{,}000}{1{,}000} * 1{,}000 = 1 * 1{,}000 = 1{,}000$$

Discussion

The Sedgewick-Rafter slide is best used when analysing cultures or high biomass blooms. As this method does not require an overnight settling period, it is rapid and can provide a quick assessment of a water sample. It has been proven to provide accurate results between 10,000 (ICES 2006) and 100,000 cells L^{-1} (McAlice 1971). The set up cost is low due to the use of a compound microscope.

The Sedgewick-Rafter slide tends to perform better with samples containing larger phytoplankton cells. Another counting method may have to be used for samples with low cell densities. It may be possible to pre-concentrate cells using a filtering/settling step when the target organism is present in low concentrations.

Plastic Sedgewick-Rafter slides tend to scratch easily and care must be taken when cleaning the cell. Scratches may hinder the accurate identification of cells. Due to the design of the Sedgewick-Rafter slide it may be difficult to use the 40X magnification. This could prove a problem in the identification of smaller (10-15 µm) phytoplankton cells. In addition exteme care must be taken to load the Sedgewick-Rafter slide correctly and avoid the introduction of air bubbles to ensure the even distribution of phytoplankton in the slide.

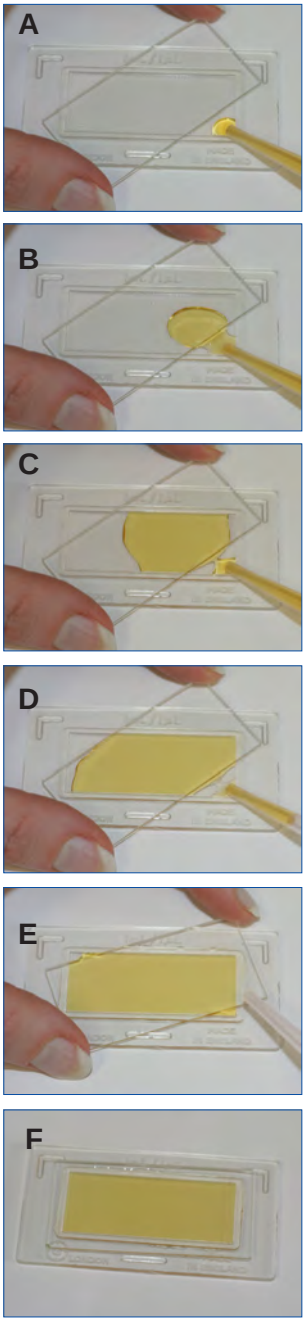

Figure 1. A – F: Loading Lugol's iodine preserved sample into Sedgewick-Rafter cell.

Palmer-Maloney counting slide

Introduction

The Palmer-Maloney counting slide method is a rapid and straightforward technique that was first employed to enumerate nanoplankton. The counting chamber is round, measures 17.9 mm in diameter, 400 µm depth and holds a volume 0.1 mL of sample. Two loading channels are located on either side of the counting slide (Fig. 2 a-d). The Palmer-Maloney slide does not have any rulings or grid. This counting slide is useful with cultures, samples with high cell densities or pre-concentrated samples. The detection level is 10,000 cells L^{-1} or 10 cells mL^{-1}(Guillard 1978).

Equipment

A standard compound microscope or an inverted microscope with 10X and 20X objectives. Phase contrast and epifluorescence capability are valuable asset to the identification of phytoplankton.

- Palmer-Maloney counting slide
- Cover glass (22mm X 22mm or 50mm X 22 mm)
- Pipettes (Pasteur or disposable) needed to dispense the sample
- A tally counter is useful when counting high cell numbers

Chemicals and consumables

- Preservative (Lugol's iodine, Formalin) (see chapter 2 for recipes)
- Alcohol for cleaning slides

Method

1 The cover glass should initially be placed over the counting chamber;

2 The preserved sample should be inverted gently about 10-20 times to ensure homogenisation. A pipette is filled with the well-mixed sample;

3 The chamber of the counting slide is then filled by gradually dispensing an aliquot of sample from the pipette into one of the loading channels (Fig. 2 a-d). It is important that no air bubbles are present in the chamber. Trapped air may be removed by slowly sliding the cover slip back then replacing it in its original position however it may be nessecary to repeat steps 1-3;

4 The slide should be left to settle for 5 minutes;

5 Counting organisms should begin at the top or bottom edge and continue until all area of the chamber is examined excluding loading channels. The centre of the loading channels are effective reference points to count half of the slide;

6 Once the count has been completed the slide and cover glass should be rinsed thoroughly with water then with alcohol and wiped clean with lint-free wipes.

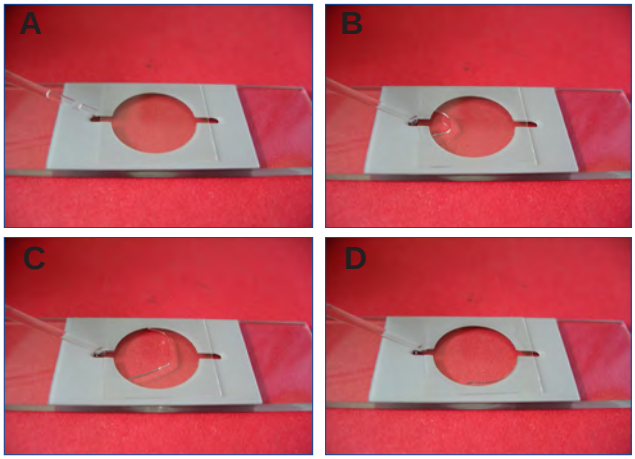

Figure 2 A –D: Loading sample into the Palmer Maloney cell

Preservation and storage

Any preserved samples can be used with this method.
Note: samples preserved in Lugol's iodine- should be kept in the dark and checked periodically for light tea colour, adding more preservative if needed.

Formulas for calculating results

Since the volume of a Palmer-Maloney slide is 0.1 mL, multiply the total count by 10,000 to obtain the number of cells L^{-1}.

For example:
Cells per Litre= total cell count * 10,000
Final count in the Palmer-Maloney slide is 200 cells; 200 * 10,000= 2,000,000 cells L^{-1}

Discussion

The Palmer-Maloney counting slide method is excellent to enumerate dense blooms, net tows, cultures or pre-concentrated samples. It is an inexpensive and rapid counting method in which the entire phytoplankton community may be observed including harmful algae species.

This counting slide is appropriate for enumerating most species but is not as useful for counting large organisms (>150 µm) or long chain-forming diatoms as these may not be distributed evenly in the sample (Guillard and Sieracki 2005).

Because of the thickness of the Palmer-Maloney slide the highest magnification objective lenses possible to use with some compound microscopes is 10X or 20X. Thus this slide is not a good choice when the proper identification or enumeration of an organism requires a higher magnification. This can be addressed by using an inverted microscope with a higher objective. The Palmer-Maloney slide may also be used with calcofluor stain and an epifluorescence microscope for thecate dinoflagellate identification.

When using the small cover slips (22 mm x 22 mm) the sample may have a tendency to evaporate. This problem can be solved by using longer cover slips (22 mm x 55 mm) or by applying parafilm to cover the loading channels. This method may be used to monitor target species that are deemed to be harmful only at very high cell concentrations.

Haemocytometer counting method

Introduction

The haemocytometer (also used for counting blood cells) is a counting slide method specially practical for cultures and extremely high concentrations of cells of small sized organisms (< 30 µm). The middle of the slide has the appearance of an "H" which separates the 2 thin silver-coloured chambers both engraved with a nine-square grid. Haemocytometers may be purchased in either 0.1 mm or 0.2 mm depth and may possess different grid subdivisions. The most common slide of this type is 0.1 mm deep with Improved Neubauer ruling (Fig. 3); each chamber holding nine 1-mm large squares separated by double or triple rulings. The volume in nine large squares is 0.0009 mL, with the 2 chambers having 18 squares with a total volume of 0.0018 mL.

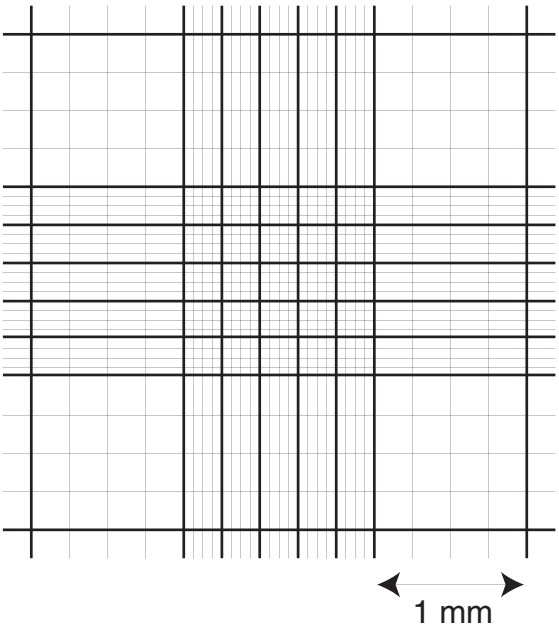

Figure 3. Drawing of haemocytometer with improved Neubauer ruling.

Equipment

- A standard compound microscope or an inverted microscope with 10X and 20X objectives, phase contrast
- Haemocytometer counting slide (Improved Neubauer rulings)
- Cover glass supplied with haemacytometer
- Pipettes (Pasteur)

Chemicals and consumables

- Preservative (Lugol's iodine, Formalin) (see chapter 2 for recipes)
- Alcohol for cleaning slides

Methods

1 A cover glass is placed over both chambers of the haemocytometer;

2 With a soft undulating motion, the preserved sample is gently inverted appoximately 10-20 times to ensure the sample is mixed thoroughly;

3 A Pasteur pipette is filled with the well-mixed sample;

4 Each chamber of the haemocytometer is loaded by holding pipette at a 30 to 45 degree angle with the open dispensing tip in the V-shaped slash, allowing the pipette tip to touch the slot then slowly expelling a drop of the liquid. The capillary action will fill the chamber with the sample. It is important to check that the liquid spreads over the silver- coloured chamber without overflowing into the moats. (Fig 4);

5 Step 2-4 is repeated to fill the other side of the chamber. Allow 2-3 minutes for cells to settle;

6 The slide should be scanned initially in the microscope to determine the counting strategy. The whole slide or a selected number of large squares should be counted to obtain a statistically significant number of cells (Andersen and Throndsen 2004). Each side of the haemocytometer slide has a grid with nine large 1 mm^2 squares which are further subdivided depending on the type of haemocytometer;

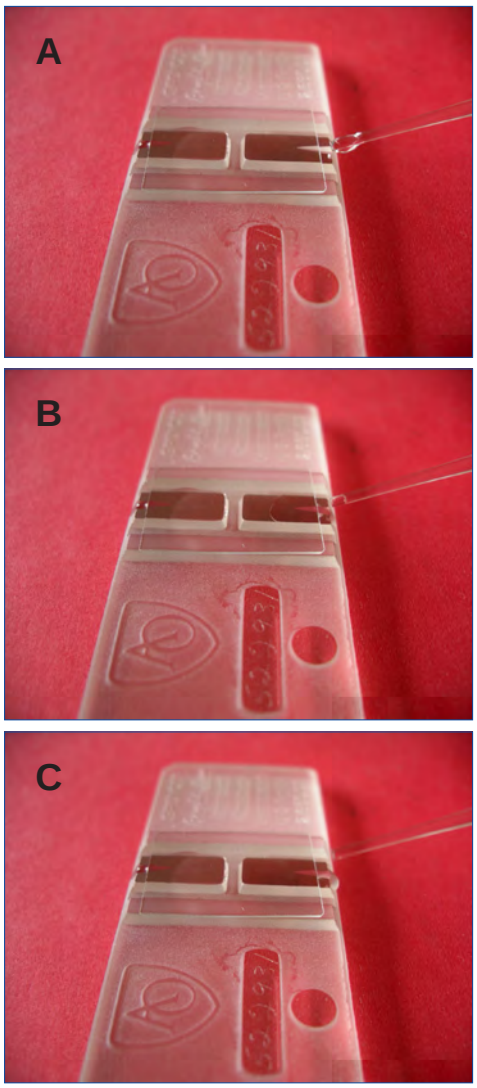

Figure 4 A-C: Loading sample into haemocytometer.

7 The number of organisms and the number of squares counted should both be noted. To avoid counting cells twice, it must be determined beforehand to include cells that touch 2 of the 4 sides of each square (i.e. the top and left side of each large square while ignoring the cells that touch the bottom and right side);

8 After the count has been completed the haemocytometer slide must be cleaned thoroughly by rinsing the slide and cover glass with running water then with alcohol, wiping clean with lint-free wipes.

Preservation and storage

Any preserved samples can be used.
Note: samples preserved in Lugol's iodine- should be kept in the dark and checked periodically for light tea colour, adding more preservative if needed.

Formulas for calculating results

Total the cells and divide by the number of large 1 mm squares counted to obtain the average number of cells per square. Multiply this average cell number by 10,000 to obtain number of cells per mL. (Alternately to obtain the number of cells per Litre, multiply the average number of cells per large square by 10,000,000).

The average number of cells per mL = average count per large square X 10,000

For example: In total 200 cells in 4 large squares are counted:

$$Average\ \ number\ of\ \ cells\ counted = \frac{200}{4} = 50$$

$$Cells\ per\ mL = 50 * 10{,}000 = 500{,}000\ \ cells\ mL^{-1}$$

Discussion

The haemocytometer counting method is excellent for counting cultures or an extremely high concentration of small cells. The slide can be used with 10X objective with the compound microscope or with 10X or 20X objectives with an inverted microscope. This method is not suitable for routine water monitoring because of the high cell biomass needed to get statistically significant numbers. It will not give an overview of the whole phytoplankton community especially organisms with a low cell density. It is not compatible with large organisms because of the shallow 0.1 mm depth of the slide. It is better to use the slide for extremely high cell estimates.

Pre-concentration of sample

Concentration of sample may be necessary when cell density is low. This can be achieved using a settlement method where a sample is poured into a graduated cylinder (of volume (*A*)) and allowed sufficient time for cells to settle (one hour for each cm height of cylinder or overnight). After settling, the water from the upper portion of the sample is gently removed and the final volume (*B*) noted. Another method involves filtering the sample through a 10 or 20 µm mesh (i.e. plankton net). The concentration factor (CF) is calculated by dividing the initial volume (A) by the final volume (B). The remaining volume should be mixed well and the instructions of the counting method followed, remembering to divide the total cell count by the *CF*.

Example:

$$CF = \frac{A}{B} = \frac{100}{10} = 10$$

The original volume (*A*) = 100 mL
Final volume (*B*) = 10 mL

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5 Filtering – calcofluor staining – quantitative epifluorescence microscopy for phytoplankton analysis

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Introduction

Identification and enumeration of thecate dinoflagellates, which include several toxic species, is frequently a time consuming exercise. Species from the genera *Dinophysis* and *Prorocentrum* (the causative organisms of diarrhetic shellfish poisoning, DSP), *Alexandrium* and *Pyrodinium* (the causative organisms of paralytic shellfish poisoning, PSP), *Ostreopsis* and *Gambierdiscus* (responsible for ciguatera fish poisoning, CFP) may cause problems to the aquaculture and fishing industries even when they occur in low concentrations. Traditionally, quantitative analysis of phytoplankton samples is performed using the well established Utermöhl sedimentation procedure (Utermöhl 1958). The Utermöhl procedure involves sedimentation of the plankton sample (5-50 mL) for a period of 8 -24 hours depending on the sample volume. The long sample sedimentation time prior to analysis and the complexities of identification of thecate dinoflagellates means that this method cannot provide a rapid result. Quantitative epifluorescence techniques, basically adapted from the acridine orange technique by Hobbie *et al.* (1977), involves filtration and staining of the organisms on polycarbonate filters. This method, which was originally described for counting of pelagic bacteria has been modified for counting of hetero- and autotrophic nanoflagellates as well as larger phytoplankton and protozooplankton organisms (see e.g. Haas 1982, Andersen and Sørensen 1986). Examples of fluorochromes used are acridine orange (Andersen and Sørensen 1986) or DAPI (Porter and Feig 1980). Apart from the DNA, these fluorochromes also stain other compounds found in cells. When working in coastal waters and shallow fjords where the pelagic biomass is high and often dominated by diatoms, quantification of thecate dinoflagellates present in low concentrations must be carried out on relative large water samples (50-100 mL). In such cases the analysis can be rendered practically impossible using either acridine orange or DAPI. This is because thecate dinoflagellates must be identified among high concentrations of other organisms such as diatoms, which also fluorescence heavily.

In this chapter, a method is presented which is based upon the quantitative epifluorescence technique using Calcoflour White M2R as a stain. The method has previously been described in Andersen (1995), Andersen and Kristensen (1995), Andersen and Throndsen (2004). Calcofluor is a specific stain for the cellulose in the thecal plates of the thecate dinoflagellates (Lawrence and Triemer 1985). The stain does not stain structures in most other pelagic organisms including the diatoms. Using this method it is possible to analyse sample volumes from 10 to 500 mL, thereby obtaining reliable estimates of thecate dinoflagellates present in low concentrations in the presence of large concentrations of diatoms.

Materials

Laboratory facilities

The method can be used in a basic laboratory. No special facilities are required.

Equipment

A filtration unit and a vacuum pump as well as an epifluorescence microscope equipped with a mercury lamp and filter set for DAPI (UV excitation (330-380 nm), emission filter (420 nm) is required, see Table 1.

Chemicals and consumables

The stain, Calcofluor White M2R (Polysciences, Warrington PA) is especially useful for qualitative as well as quantitative analysis of thecate dinoflagellates because it stains the cellulose in the thecal plates of dinoflagellates and not other plankton organisms or detritus.

Calcofluor White M2R is a fluorescent brighter. The chemical formula of Calcofluor White M2R is: $C_{40}H_{42}N_{12}O_{10}S_2Na_2$. Calcofluor White M2R can be stored at room temperature.

Table 1. Equipment and consumables required for the quantitative epifluorescence method using Calcofluor White M2R.

- Calcofluor White M2R or a similar product
- A 10 mL glass bottle
- Polycarbonate membrane filters (pore size 5 µm)
- Paraffin oil
- Neutral Lugol's iodine
- A filtration unit
- A vacuum pump
- Glass microscope slides
- Cover slips (24x24 mm)
- An epifluorescence microscope equipped with a mercury lamp and filter set for DAPI (UV excitation, 330-380 nm, emission filter, 420 nm)

Method

How to make the Calcofluor working solution

Add approximatley 2 µg Calcofluor to 10 mL of distilled water in a clean acid rinsed (5% HCl) glass bottle. The Calcofluor will dissolve immediately, and the working solution is ready to use. If the glass bottle is not completely clean the Calcofluor may precipitate and the solution can not be used.

The working solution of Calcofluor does not require preservation. It can remain viable for a few days to several weeks at room temperature in the laboratory.

The fundamentals of

The filtering - calcofluor staining - quantitative fluorescence microscopy

Scope
Qualitative and quantitative analysis of thecate dinoflagellates.

Detection range
Detection range is dependent on the volume of sample filtered. Counting all of the cells from a 200 mL sample will give a detection limit of 5 cells per Litre.

Advantages
Preparation time is short. Specific identification of thecate dinoflagellates is feasible as the staining makes the relevant morphological features visible. Preparations can be stored for analysis or re-examination for weeks/months.

Drawbacks
This method is limited to analysis of thecate dinoflagelllates only. This means that other methods will have to be used if the rest of the phytoplankton community needs to be studied.

Type of training needed
A basic knowledge of light and epifluorescence microscopy is needed. Analysis requires continuous training over years with in-depth knowledge of taxonomic literature.

Essential Equipment
Epifluorescence microscope with UV excitation
Filtration unit incl. pump.

Equipment cost*
20000-50000 US$ (see Appendix to this chapter for details).

Consumables, cost per sample**
Less than 1-2 €/$1-2.

Processing time per sample before analysis
App. 15 minutes for filtration and mounting of filter.

Analysis time per sample
Depending on the number of species to be quantified and volume filtered. A routine analysis of 5-10 species requires approx. 15 min. per sample, excl. reporting/database handling of results.

Sample throughput per person per day
10-20.

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. The stain Calcoflour is for laboratory use only. Caution: Avoid contact and inhalation! The relevant health and safety guidelines should be followed.

*service contracts not included
**salaries not included

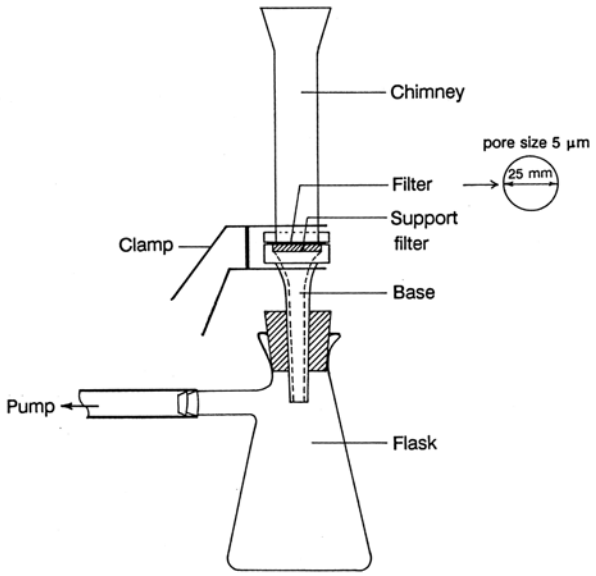

Figure. 1. The filtration equipment used to concentrate phytoplankton on polycarbonate membrane filters for quantification using epifluorescence microscopy.

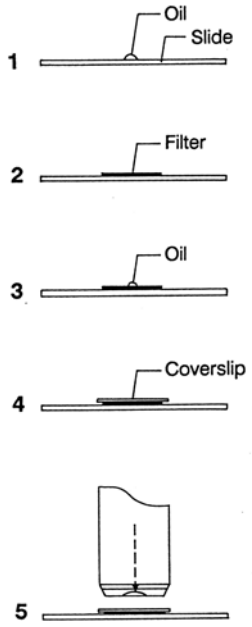

Figure. 2. How to mount the polycarbonate filter on a slide for observation using epifluorescence microscopy.

Sample preparation

A known volume of sample, fixed using neutral Lugol's iodine, is vacuum filtered using a polycarbonate filter with a pore size 5 µm (Fig. 1). Filters with larger or smaller pore sizes can be used depending upon the organisms of interest.

When approximatley 1 mL of sample is left in the filter chimney, 0.2 mL (3-4 drops) of Calcofluor White M2R working solution is added and filtration is continued until the filter is dry (Table 2).

Note that Calcofluor White M2R will only work at a neutral pH (= 7). In more acidic samples Calcofluor will precipitate which will interfere with the identificaiton and enumeration of dinoflagellate cells. If the sample is fixed using acidic Lugol's iodine, the sample must be neutralised prior to the addition of the Calcofluor. To achieve this, the filtration should be stopped when there is approximatley 0.5 mL of sample left in the filtration chimney. The filter should then be washed by adding approx. 2-5 mL of filtered seawater fixed in neutral Lugol's iodine. The filtration procedure is then continued as described as described in Table 3.

The dry filter should be mounted on top of a drop of paraffin oil on a microscope slide. Another drop of paraffin oil is then placed on top of the filter and a cover glass is mounted on top of the paraffin oil (Fig. 2). The filter can then be analysed using epifluorescence microscopy, using UV excitation (330-380 nm) and an appropriate emission filter (420 nm). For routine use an OLYMPUS BH-2 microscope equipped with a mercury lamp (100 W) or a similar microscope is appropriate.

Thecate dinoflagellates can be identified and enumerated, either by counting the whole surface of the filter or selected fractions of the filter surface. Large organisms (diameter >20 µm) such as cells from the genus *Dinophysis, Prorocentrum* or *Alexandrium,* can be counted using 100X magnification. Higher magnifications can be used when it is necessary to see the morphology of the thecal plates to allow identification to species level.

The prepared slides for quantitative analysis can be stored in a refrigerator (< 5°C) for weeks with no/very little loss of cells or fluorescence.

Trouble shooting

The most frequent problems encountered when working with the quantitative Calcofluor method are:

1 The filter set on the epifluorescence microscope does not work with Calcofluor White M2R;
2 The pH of the sample to be analysed is not 7;
3 The working solution of Calcofluor has precipitated and the solution looks milky.

Preservation and storage

Samples to be analysed with this method should preferably be fixed using neutral Lugol's iodine. If samples are to be stored for more then a few days the samples should be stored in brown glass bottles and stored in the dark. Samples stored according to these guidelines can be kept for months/years, however, it must be ensured that there is sufficient neutral Lugol's iodine in the sample to maintain the perservation. If the sample has the colour of weak tea there is sufficient Lugol's present. If the sample is clear, more neutral Lugol's iodine must be added.

Counting procedure and calculation of concentrations

Initial analysis of the sample can be performed using a low magnification such as 100X. The performance of the Cal-

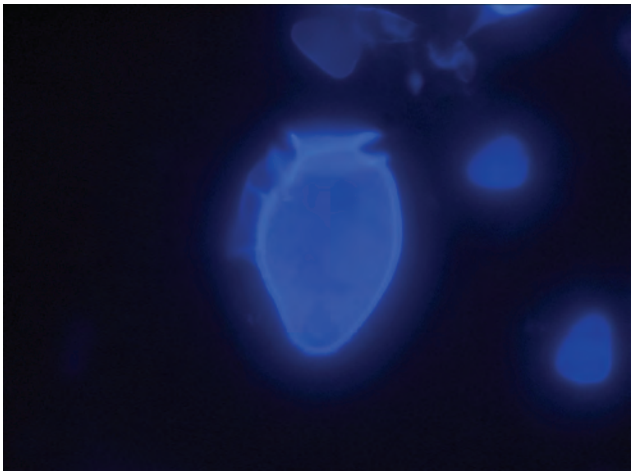

Figure 3. *Dinophysis* sp. stained with calcofluor as seen in the epifluorescence microscope. The cell height is approximately 50 µm.

cofluor stain must first be assessed by establishing that the thecate dinoflagellates light up blue on a dark background. After this has been checked, the sample analysis can be performed.

The counting strategy employed depends on the number of cells on the filter. It is preferable to count the entire surface of the filter. If there are a high number of cells on the filter, subsampling or counting only a fraction of the filter (half of the filter surface or diagonals), can be performed.

Calculation of cell concentrations:
To calculate the concentration (cells L^{-1}) of the different species in your preparation you must know:

V = Volume of sample concentrated on the filter (mL).
B_a = Area of the filter (mm^2).
B_c = Area of the part of the filter counted (mm^2).
N = Number of cells counted for the species of interest.

The conversion factor (CF):

$$CF = \frac{B_a}{B_c}$$

The concentration of the species C (cells mL^{-1}) is then:

$$C = N * \frac{CF}{V}$$

The conversion factors must be calculated for each filtering unit and microscope as well as for each combination of subsampling area and magnification (Table 4).

Examples of calculations of concentrations using the conversion table are presented in Table 5.

Table 2. Summary of how to prepare samples preserved with neutral Lugol's iodine for the quantitative epifluorescence method using Calcofluor White M2R.

1. Measure the required sample volume using a graduated cylinder;
2. Add the sample to the filtration unit;
3. Turn on the vacuum pump (maximum pressure = 200 mmHg);
4. Turn off the vacuum pump when there is approximately 1 mL left in the filtration chimney;
5. Add 3-5 drops of CalcoFluor working solution (concentration 2 mg L^{-1});
6. Turn the vacuum pump on again and filter until the filter goes dry;
7. Remove the filter from the chimney and dry the back of it gently on a tissue to remove 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 x 24mm);
9. Observe your preparation using an epifluorescence microscope.

Table 3. Summary of how to prepare samples preserved with acidic Lugol's iodine, formaldehyde or gluteraldehyde for the quantitative epifluorescence method using Calcofluor White M2R.

1. Measure the required sample volume using a graduated cylinder;
2. Add the sample to the filtration unit;
3. Turn on the vacuum pump (maximum pressure = 200 mmHg);
4. Stop the filtration when there is about 1-0.5 mL left in the chimney;
5. Wash the filter by adding approx. 2-5 mL of filtered seawater fixed in **neutral** Lugol's iodine and turn on the vacuum pump;
6. Turn off the vacuum pump when there is approximatley 1 mL left in the filtration chimney and add 3-4 drops of the Calcofluor working solution (concentration 2 mg L^{-1});
7. Turn on the vacuum pump again and filter until the filter goes dry;
8. Remove the filter from the chimney and dry the back of it gently on a tissue to remove 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 (24x24mm);
9. Observe your preparation using an epifluorescence microscope.

Table 4. Example of a calibration table used for calculating concentrations of microalgae using epifluorescence microscopy (filter area = 189 mm^2. Note: CF is the conversion factor to be applied when the count of cells in e.g. one diagonal window is to be calculated to the concentration on the total filter surface.

Magnification	Window count Window area (mm^2)	Diagonal window count Diagonal window area (mm^2)
40X	4.08	31.52
100X	0.66	12.64
200X	0.16	6.2
	CF: window	**CF: diagonal window**
40X	46.3	6.04
100X	286	15.1
200X	1181	30.5

Table 5. Examples of calculations of cell concentrations using the conversion table.

Example 1 (counting the entire filter)
Volume of sample concentrated on the filter = 100 mL
Counts (entire filter area) = 50 *Dinophysis acuminata*

Calculating cell concentration
(50/100) = 0.5 cells mL^{-1} = 500 cells L^{-1}

Example 2 (counting one diagonal window)
Volume of sample concentrated on the filter = 100 mL
Counts (Diagonal window 100X) = 50 *D. acuminata*
CF = 15.1

Calculating cell concentration
(50 x 15.1)/100 = 7.5 cells mL^{-1} = 7500 cells L^{-1}

Discussion

The method described is excellent for the rapid processing of samples, for example as required in many toxic phytoplankton monitoring programmes. Low concentrations of thecate dinoflagellates are easily detected in the presence of high pelagic biomass (e.g. diatoms). This is particularly relevant for concentrated net tow samples. It is possible to identify thecate dinoflagellates to species level because of the easy recognition of the thecal plates of taxonomical importance which can not always be recognised using the Utermöhl procedure using Lugol's iodine preservation.

Samples preserved with neutral Lugol's iodine produce excellent slide preparations. There is no requirement to use more toxic preservatives like formaldehyde or glutaraldehyde. The fluorochrome Calcoflour White MR2 is considered to be of low toxicity.

Depending upon the organisms to be quantified, the described method can easily be modified by using other filters such as low cost glass fiber filters (GFC filters). Large and robust species e.g. from the genus *Ceratium*, occurring in low concentrations, can be investigated using large volumes of water (500 mL) and GFC filters with excellent results.

The procedure can be used to separate auto- from heterotrophic thecate dinoflagellates on the basis of the presence or lack of chlorophyll and other pigments by switching between appropriate filter sets (Lessard and Swift 1986, Hallegraeff and Lucas 1988, Carpenter *et al.* 1991).

It has been observed that species from the genus *Alexandrium* can break/implode (maximum 10% of the cells) during sample preparation. Breakage of cells appears to occur in cases where the filtration time is prolonged as a result of the filter blocking due to high sample biomass. This problem may be common to all filtration based methods. It is strongly recommended that this problem be investigated more fully. It is suggested that the loss of cells can be minimised by adjusting the combination of filter pore size and the volume of sample filtered thus reducing the total number of particles in the sample.

When counting, one has to be aware that empty thecae ("ghost" cells) can be hard to distinguish from cells which were viable when sampled. When counting viable cells, the "ghost" cells can lead to a slight overestimation of the concentration of viable cells. "Ghost" cells can be confirmed by switching between filter sets, e.g. to blue light excitation, which will reveal the cell content of the thecae under examination.

The resulting high contrast between thecate dinoflagellates and the dark background common in this technique as well as the lack of interference from other organisms such as diatoms means that this technique may be very useful in combination with image analysis to develop automated procedures for computerised identification and counting of specific thecate dinoflagellates.

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Appendix

Table 1. Equipment and costs

Item	**Price**
Epifluorescence microscope with UV excitation	20000-50000 US $
Mercury burner	100 US $/approx. 1200 samples, 0,08 US $/sample
Filtration unit incl. pump.	1000-1500 US $
Polycarbonate filter	0,5-1 US $/sample
Calcofluor (for a life time)	50-100 US $/approx 5.000 samples, 0,02 US $/sample
Neutral Lugol´s (1 Litre)	approx. 50 US $ /approx. 500 samples, 0,10 US $/sample
Slides and coverslips	0,05 US $/sample
Paraffin oil	0,05 US $/sample

6 Filtering – semitransparent filters for for quantitative phytoplankton analysis

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Introduction

Phytoplankton species such as large dinoflagellates are often present in the water column in low cell densities. Thus, samples must first be concentrated in order to accurately quantify these species. One method for concentrating phytoplankton cells in water samples is the use of semitransparent membrane filters (Fournier 1978). In this method water samples are directly filtered on a membrane filter which then is placed under the microscope for the identification and enumeration of phytoplankton cells.

Materials

Laboratory facilities

No specialised laboratory facilities are necessary for this method. Water samples using preservatives with potential harmful effects should be handled following the appropriate health and safety procedures.

Equipment

The following equipment is required:

- Compound light microscope, with at least 100X and 200X times magnification.
- Filter manifold and vacuum pump (handpump or electric) with manometer to gauge the pressure.
- Forceps for handling filters.

Chemicals and consumables

The following consumables are required:

- Semitransparent membrane filter (e.g. PALL GN-6). The filters are availaible in a variety of diameters and pore sizes. A filter diameter of 25 mm and a pore size of 0.45 µm is recommended for use in this method.
- Glass microscope slides and coverslips.
- Lugol's iodine solution is recommended.
- Glass bottles (dark) for sampling, transport and storage of water samples.

Methods

The recommended preparation procedure is as follows:

1 The filtration manifold is assembled and the vacuum pump tested (Fig. 1);
2 Using forceps, the filter is placed in the filter holder. The funnel and holder are connected securely to prevent leakage;
3 The preserved sample is gently agitated, by inverting the sample bottle at least 10 times;
4 The volume to be filtered is measured using a graduated cylinder. The most appropriate volume for analysis may vary with location and time of year. Too many organisms on the filter will prevent the identification of some species. A guiding volume could be 25 mL for estuaries with high production, 50 mL for inshore stations and 100 mL for offshore stations;
5 The contents of the graduated cylinder are transferred into the funnel above the filter;
6 A gentle vacuum is applied for filtration, less than 1/6 atmosphere (125 mm Hg), using a hand or electric pump with manometer. A low vacuum pressure prevents destruction of and minimises distortion of fragile cells.
7 Filtering continues until the filter is "dry";
8 The filter is removed from the filtration apparatus using forceps and placed on a microscope slide;
9 The slide is analysed using a compound light microscope at the desired magnification. The concentration of phytoplankton is calculated based on the number of cells counted on the whole filter and the volume of sample initially filtered.

Figure 1. Filtration manifold and hand held vacuum pump.

To calculate the concentration (cells L^{-1}) of the different species in your preparation you must know:

V = Volume of sample concentrated on the filter (mL).
B_a = Area of the filter (mm^2).
B_c = Area of the part of the filter counted (mm^2).
N = Number of cells counted for the species of interest

The fundamentals of

The filtering - semitransparent filters method

Scope
Concentration and enumeration of phytoplankton, particularly large and more physically robust species.

Detection range
10-50 cells per Litre, depending on volume filtered.

Advantages
This is a rapid and flexible method for the concentration of phytoplankton using standard easy to use laboratory equipment. The volume to be analysed can be easily adjusted depending on cell density.

Drawbacks
Fragile cells can be destroyed during the filtration process.

Type of training needed
No special training is required for the preparation of samples. Taxonomic competence required for identification and enumeration.

Essential equipment
Compound light microscope, filtration manifold, vacuum pump, forceps, semitransparent filters and microscope slides.

Equipment cost*
Microscope: Depending on manufacturer and type, from 4000 € (6000 US $)
Filtration manifold: from 250 € (320 US $)
Vacuum pump: hand held from 180 € (240 US $)

Consumables, cost per sample**
Cost for filters and glass is approximately 1 € (1.5 US $).

Processing time per sample
Preparation time for the filters is 5 to 10 minutes, depending volume filtered, amount of phytoplankton and filtration volume.

Analysis time per sample
Analysis time is about 30 minutes for target species, depending on skill, quantity of phytoplankton cells and number of target species.

Sample throughput per person per day
10-12 samples per day.

Health and Safety issues
Some fixatives that are used for phytoplankton preservation could be potential harmful. Appropriate health and safety procedures must be followed at all times. Continual microscope work could result in strain injury. It is important to incorporate breaks into the daily analyses time. Ergonomic adjustments to the microscope and the working place is recomended.

*service contracts not included
**salaries not included

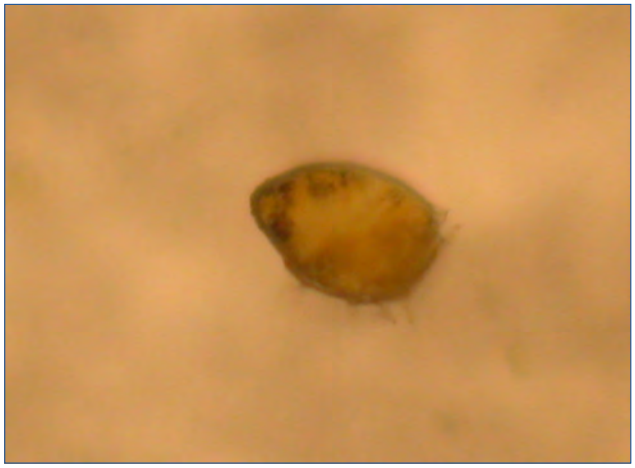

Figure 2. *Dinophysis* sp. observed on a semitransparent filter.

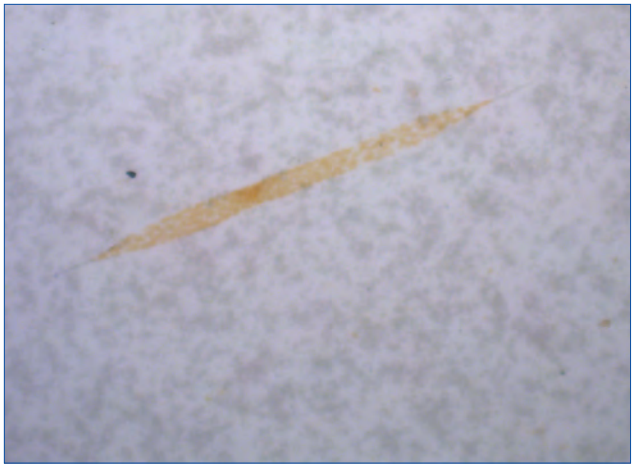

Figure 3. *Rhizosolenia* sp. observed on a semitransparent filter.

The conversion factor (*CF*):

$$CF = \frac{B_a}{B_c}$$

The concentration of the species *C* (cells mL^{-1}) is then:

$$C = N * \frac{CF}{V}$$

The conversion factors must be calculated for each filtering unit and microscope as well as for each combination of subsampling area and magnification.

Preservation and storage

Water samples can be preserved with neutralised formaldehyde or neutral Lugol's iodine solution. Formaldehyde should be used with care because of its toxicity and potential to trigger allergic reactions. Neutral Lugol's iodine solution is recommended for this method. If necessary, the brownish coloration of the algae caused by this preservative can be removed by oxidising the Lugol's iodine using a few drops of sodium thiosulfate per mL (3 g $Na_2S_2O_3$ in 100 mL water). When using Lugol's iodine solution the water samples should be stored in dark glass bottles in the dark. Samples should retain the colour of 'weak tea'. The fixation of archived samples should be checked every third month and additional Lugol's iodine should be added if the sample has lost its colouration. Properly fixed samples can last for years with appropriate maintenance.

Once a sample has been filtered it should be analysed immediately. It is possible to store samples for one day, but this may impede taxonomic identification. If samples are to be stored the filter should be kept moist. A drop of filtered seawater should be added to the filter on the microscope slide. A cover slip should be placed on top of the filter and gently pressed downward. The microscope slide is then placed in the refrigerator to reduce any evaporation. If the filter has dried a new drop of seawater should be added without lifting the cover slip prior to analysis of the sample on the microscope.

Discussion

Filtering with semitransparent filters is a rapid method for concentrating phytoplankton samples before enumeration using light microscopy. It is a flexible method as the volume filtered can be easily altered depending on the target species for analysis. The detection limit can be improved by increasing the volume filtered. The equipment needed for this concentration method is standard laboratory equipment and relatively inexpensive to purchase.

The main advantage of using the filtration method is the short handling time from when the water sample arrives in the laboratory to when it is analysed. The pore size of the filters determines the size of the cells retained. If only a defined size fraction of the phytoplankton is of interest, using filters with a specific pore size could be advantageous as this reduces the amount of background non target particles. The equipment needed for the method is in most cases relatively straightforward, compact and easy to use in the field.

The method has some disadvantages compared to other methods. Some difficulties may arise in the taxonomic characterisation of phytoplankton cells on filters as they cannot be physically manipulated to change their orientation to allow better examination of morphological features. Any non random distribution on the filter could interfere with the results if only a portion of the filter is analysed. The main disadvantage of this method is that cells can become distorted, or destroyed during the filtration process. This method is not recommended for fragile and delicate phytoplankton species, e.g. Haptophytes and Chrysophytes, since these species would be unidentifiable after the treatment. The semitransparent filter method favours the more robust species, e.g. diatoms and thecate dinoflagellates (Figs. 2 and 3).

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7 The filter - transfer - freeze method for quantitative phytoplankton analysis

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Introduction

Precise and accurate identification and enumeration of phytoplankton cells in field samples is fundamental to the maintenance of time-series data on distribution and abundance of taxa, as well as for biological oceanographic research and plankton surveillance programmes, e.g. for harmful algal blooms. Such counting methods are also required for cell enumeration in unialgal or multialgal cultures for laboratory or mesocosm experiments on phytoplankton. In this sense, the term "phytoplankton" is used loosely to include all eukaryotic microalgae, protists, cyanobacteria and unicellular benthic and epiphytic taxa, including cysts and other resting stages. Both "classical" methods based upon microscopic observations of morphological features of whole cells and molecular methods (nucleic acid hybridisation, antibodies, lectins, etc.) are now available for comparison (Godhe *et al.* 2007).

Although the filter-transfer-freeze (FTF) technique is considered among the classical approaches for cell counting and identification, it is not specifically a cell enumeration method, but rather a means of cell concentration, collection, and transfer for counting by alternative means. When applied correctly, the accuracy and reproducibility of cell counts performed by the FTF technique is more a function of the subsequent counting and identification methods than of the FTF procedure itself. The FTF method was introduced more than two decades ago (Hewes and Holm-Hansen 1983), but in spite of its simplicity and proven effectiveness, the method has not been widely employed. This is regrettable because the FTF method can be applied for both critical taxonomy (with some caveats) and for rapid but superficial analysis of phytoplankton samples. The original method was designed with respect to nanoplankton and indeed appears to work best for taxa in the size range of ca. 5 – 200 µm diameter. Smaller cells tend to get lost to the filter and larger organisms do not transfer well. Nevertheless, this size-range embraces most of the diatoms and nanoflagellates of interest and chain-formation does not markedly decrease transfer efficiency from the filter to slide. The method has been rigorously tested for use in field monitoring of phytoplankton in the Gulf of St. Lawrence region of Atlantic Canada (J. Smith and K. Pauley, Dept. of Fisheries and Oceans, Canada, unpublished manual) and in comparison with alternative methods for counting cells in cultures of the marine dinoflagellate genus *Alexandrium* (Rafuse 2004). Consolidation of the methodological variants in this chapter may assist in wider dissemination of this rather neglected method for phytoplankton cell enumeration.

Materials

Equipment

- Electric or manual vacuum pump (preferably with vacuum gauge)
- Filtration apparatus (funnel, clamp, frit, rubber stopper, connecting rubber or Tygon tubing) – single or multiple as required
- Vacuum trap flask (if connected to electric pump)
- Waste disposal flask (for filtered fixatives/preservatives)
- Freezer (-20 ˚C), dry ice or cold block (prefrozen)
- Digital or mechanical cell counter (optional)
- Research microscope equipped with bright-field, phase-contrast or Normarski optics
- Standard glass microscope slides
- Glass cover slips (24 x 24 or 24 x 50 mm)
- Watch glass
- Polycarbonate membrane filters (25 mm); Poretics™ or Nuclepore™ (pore size: 3 µm or dependent upon size distribution of taxa of interest)
- Pasteur pipettes with rubber bulb
- 0.5 or 1.0 l plastic squirt bottle
- Millipore-type flat forceps
- Grease pencil (optional)
- Source of filtered (0.22 µm) seawater
- Fixatives/preservatives (if desired)
- 95% or 70% ethanol (for cleaning)

Laboratory facilities

The FTF method and subsequent microscopic analysis require no sophisticated facilities. The technique can be performed even in a rudimentary "laboratory", at dockside or on board ship, if a means of quick freezing of the slide is available. In the absence of a laboratory freezer, the slide can be frozen upon dry ice, liquid nitrogen (carefully!) or upon an aluminum or plastic cold block that has been prefrozen at -20 to -80 ˚C and maintained in a well-insulated container. The ba-

Figure 1. Filtration and vacuum apparatus used in the FTF method.

The fundamentals of

The filter - transfer - freeze method

Scope
Appropriate for fixed, labelled, or unfixed planktonic chain-forming or unicellular organisms.

Detection range
Detection range is dependent on the volume filtered. Best precision and counting accuracy is achieved with transfer of 200 – 400 cells per taxon of interest to the slide surface.

Advantages
Can be applied for enumeration of cells prepared with a variety of different preservation and labelling methods or from fresh samples. The method can accommodate alternative microscopic techniques: bright field, phase-contrast, Normarski, and epifluorescence methods. The preparation is extremely simple and rapid (minutes) and is limited only by the filtration time.

Drawbacks
Potential loss of cells in the transfer process and some morphological distortion of delicate specimens is possible. The filtration and microscopic method is normally applied serially, i.e. one sample at a time, although in principle multiple filtration funnels could be used to filter samples in parallel. Other limitations of the method are generic to all optical microscopic techniques (resolution limit, operator error in identification, etc.).

Type of training needed
Only a simple practical demonstration of the filtration and transfer method is required – this method can be mastered in <1 hour with an expected unsuccessful transfer occurring only in the first five attempts. Phytoplankton identification requires continuous training over years with in-depth knowledge of taxonomic literature.

Essential Equipment
Vacuum filtration apparatus with pump, optical microscope and a source of rapid freezing (-20 ºC freezer, dry ice, or cold block).

Equipment cost*
100 – 2000 € (excluding microscope). Estimated cost for acceptable microscope ranges from 3,000 € for a simple bright field or student-grade microscope to >60,000 € for a fully equipped research microscope with Normarski or epifluorence capabilities.

Consumables, cost per sample**
Less than 2 €/2 US $ (determined mostly by the cost of the filter).

Processing time per sample before analysis
Varies with the volume of water filtered, but typically <5 minutes.

Analysis time per sample
Limited by the degree of scrutiny required and the complexity of the sample. For accurate and precise counts from monocultures or simple mixtures <10 minutes would be expected, whereas for complex plankton matrices from dense field samples up to 1 hour per sample may be required. These timings are dependent on the skill of the analyst.

Sample throughput per person per day
10 – 50 samples per day (8 working hours), depending upon the cell concentration and sample complexity.

No. of samples processed in parallel
Up to 12 filtrations may be carried out simultaneously, but microscopic observations must be in series.

Health and Safety issues
Determined only by the toxicity of the fixative and labelling components (if any) used for the preparation of the samples. Appropriate health and safety procedures must be followed at all times. Continual microscope work could result in strain injury. It is important to incorporate breaks into the daily analyses time. Ergonomic adjustments to the microscope and the working place is recomended.

*service contracts not included
**salaries not included

Figure 2. Removal of filter from microscope slide using forceps.

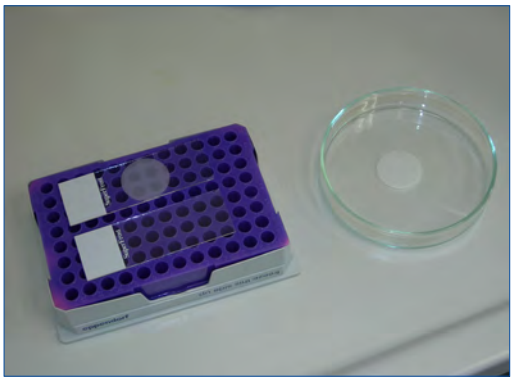

Figure 3. Microscope slide after the filter has been removed. The filter is retained in the Petri dish

Figure 4. A drop of seawater is added to the microscope slide containing the sample and a coverslip added.

sic requirements are approximately 1 m^2 free bench space for vacuum pump, filtration apparatus, in addition to space for mounting of a standard microscope.

Methods

Sample collection, fixation, storage and preparation for microscopic analysis have been well described in the UNESCO Manual on Harmful Marine Microalgae in the chapter by Andersen and Throndsen (2004) on estimating cell numbers. This reference should be consulted for general procedures. Only specifics relevant to the FTF method are provided here as follows:

1 Prepare a clean glass microscope slide by outlining a circular filter-separator (25 mm) with a black grease pencil. This step may be omitted as it does not always provide an advantage for corralling the cells on the slide;

2 Mount a polycarbonate membrane filter (25 mm diameter) onto the the filtration apparatus and ensure that the filter remains centred when clamping or threading the filtration funnel (Fig. 1);

3 Filter a suitable volume of plankton sample (must be determined empirically after initial trials and rough counting of taxa of interest) under gentle vacuum (<200 mm Hg) but at a suitable flow rate (ca. 50 mL min^{-1});

4 Continue filtration until a few drops are left in the funnel, then turn off pump and allow the residual vacuum to draw the filter just to the point of dryness. Release vacuum slowly;

5 Remove the filtration funnel and with flat forceps (Millipore™- type) carefully lift off the filter, invert it onto the centre of the prepared glass slide (in the centre of the grease pencil ring if this step was followed);

6 Place the glass slide onto a cold flat surface (dry ice or cold block in the freezer) filter side up and allow the filter to freeze completely to the slide (usually two minutes is sufficient time);

7 Remove the glass slide from the cold surface or from the freezer, and place on a flat counter area with the filter side up;

8 If the filter slide is crackling frozen, wait a few seconds until the frost just begins to disappear. With a flat forceps, grasp the edge of the filter and with a smooth rolling motion of the hand, peel back the filter parallel to the specimen surface until the filter is free from the slide. A deposit of material should be apparent at the centre of the slide (Fig. 2). Retain the filter (shiny side up) in a watch glass or Petri dish for further observation (Fig. 3);

9 Add a drop or two of filtered seawater (0.22 µm) with a Pasteur pipette to the sample at the centre of slide. Place the glass coverslip (25 x 50 mm) carefully upon the glass slide (Fig. 4);

10 Identify and count the cells by viewing the entire contents trapped inside the grease chalk circle or under the entire cover slip. Cells should be counted in zigzag transects under the appropriate magnification for identification;

11 Calculate cell numbers as the total cells counted on the slide per unit volume of sample filtered (assumes 100% transfer efficiency from the filter to the slide).

Useful notes on the application of the method

1 The optimal filter pore size must be determined by the cell size of the taxa of interest, the concentration of the suspended particulates in the seawater sample and the volume of seawater to be filtered. In general, use the largest available pore size for the filter (to maximise flow through and minimise clogging) that will retain all of the key taxa. For nanoplankton samples, 3 or 5 µm pore size is usually a good compromise;

2 A drop or two of filtered seawater can be useful in seating the filter on the filter apparatus. Make sure the filter is always mounted in the same orientation, usually shiny-side up for most types (but check!);

3 It is not usually feasible to count more than a few hundred cells of a given taxon on a single slide, but counts must be sufficiently high (see Andersen and Throndsen 2004) to avoid having to count many replicate slides for statistical validity. The sample must be thoroughly homogenised in the bottle by a gentle end-to-end and side-to-side rolling motion between the hands before filtration;

4 A wet filter will result in the loss or mobilisation of cells on the filter as it is removed from the funnel. This is likely to be the largest source of error in the method. On the other hand, sucking the filter under high vacuum to complete dryness or for a prolonged time will embed the cells in the membrane and they will not transfer efficiently from the filter;

5 Make sure that there are no pleats or folds in the filter and that the entire surface is in good contact with the slide;

6 Perform a cursory examination of the upper filter surface for residual cells that were not transferred. This can be done quickly with a stereo-microscope or a standard microscope under low power (40X). If more than a few cells are present, the filtration procedure must be repeated;

7 This procedure is designed for immediate observation of specimens without archiving. Techniques for preparing semi-permanent mounting and embedding with various preparations of glycerol and embedding medium for later taxonomic analysis may be consulted in Hewes and Holm-Hansen (1983);

8 Either of two techniques can be used for placing the cover slip: 1) gently lowering one end of the coverslip until contact is made with the water droplet, then letting surface tension act as the cover slip is lowered at an angle; or 2) the "bombardier principle", whereby the cover slip is dropped gently from just above the water droplet. Take care to avoid bubbles under the coverslip.

Preservation and storage

The FTF method can be applied to microscopic analysis of plankton samples directly from seawater, since filtration first immobilises and quick-freezing kills the cells. Cells frozen to the filter may be stored in the freezer for several hours (or even overnight) without apparent damage or effect on subsequent transfer. Nevertheless, for archival purposes, fixation and/or preservation of cells may be desired to reduce decomposition and to maintain morphology for future identification and enumeration. Aldehyde preservatives affect steric configuration by cross-linking of proteins via multiple interaction with various amino acid residues and even peptide bonds (Shi *et al.* 2000), whereas ethanol is a coagulant fixative causing limited and unstable cross-linkages. Although exhaustive comparative trials have not been conducted specifically for the FTF method, all commonly used preservatives and fixatives (Keller and Manak 1993, Amann 1995, Cañete *et al.* 2001), including Lugol's iodine solution, formulation of gluteraldehyde, formalin or paraformaldehyde (PFA) in various buffer solutions, as well as saline-ethanol mixtures for application of molecular probes are generally compatible with this method.
Details of fixation and preservation are beyond the scope of this chapter, but a few general observations here with respect to the FTF method are relevant. Lugol's iodine solution has been traditionally used to preserve microalgae for counts via Utermöhl settling chamber method (see Chapter 2, this volume) and as such can be employed also for the FTF technique. The disadvantages of this solution are that it strongly colours cells so that autotrophic and heterotrophic cells cannot easily be distinguished and cells cannot be readily stained for epifluorescence microscopy. Decolourisation (i.e. with thiosulphate) is possible but often results in a loss of morphological details and even cell lysis. Lugol's solution is particularly effective with highly silicified structures, e.g. most diatoms and silicoflagellates, but less so for naked and thecate flagellates for which details are often obscured.

Gluteraldehyde is excellent for fixation and preservation of structures, particularly for subsequent analysis by SEM or TEM, but is relatively expensive and highly toxic. Use of gluteraldehyde is therefore discouraged for the FTF method, which does not usually yield the highest quality samples for electron microscopy in any case. Other aldehydes fixatives are preferred, for a compromise preservation of siliceous, calcareous and cellulosic structures of phytoplankton. Acidic formalin preparations can be very destructive to calcareous structures, such as those of coccolithophorids.

A high quality universal preservative for most phytoplankton samples based upon buffered paraformaldehyde (PFA) has been extensively tested with the FTF method. This formulation stabilises most cell structures and provide a robust resistance to filtration and freeze-thaw damage. The recipe is therefore given here as follows:

Preparation of 10% PFA:

1 Add 100 g of PFA powder to 800 mL dH_2O (or a suitable buffer);
2 Heat to about 60-80 ºC with constant magnetic stirring;
3 Add NaOH (1N) gradually until PFA is just completely dissolved (do not add too much!);
4 Let the solution cool to room temperature
5 Adjust to desired pH using NaOH or HCl (1N);
6 Add H_2O (or buffer) to make up to 1000 mL.

For most plankton samples, acidity should be adjusted to pH 4 and final concentration in the sample should be 1 – 2%. Thereafter, samples can be archived for many months prior to filtration for FTF microscopic analysis.

Discussion

The FTF technique has some of the advantages and drawbacks of other filtration-based techniques. Nevertheless, the problems cited for direct counting of cells upon filters (morphological distortion of cells, poor contrast, low resolution, difficulty in applying stains, etc.) (Hewes and Holm-Hansen 1983) are effectively eliminated in the FTF method. Relative to other classical counting methods, such as the Uter-

möhl settling chamber method, the FTF technique has the advantage that there is no time-lag for sedimentation, with attendant concerns about physical disturbance of the setting regime (e.g., on board ship). Counts can be performed with a standard research microscope rather than with a less common inverted optical system. As a cell concentration and transfer technique, the FTF method can be successfully combined with other methods for critical taxonomy by epifluorescence microscopy, such as the calcofluor staining method for the cellulose plates of dinoflagellates, or application of fluorescence probes for nucleic acids or antibody targets. In the fluorescence approach, the appropriate stains can be applied at the edge of the cover slip and drawn across the sample by applying a laboratory tissue at the opposite corner of the cover slip.

Furthermore, FTF preparation time is only a few minutes, with filtration as the rate-limiting step. This makes it easy to adjust the cell concentration on the counting slide for optimal accuracy and precision of counting (typically 200 - 400 cells per taxon of interest) by varying the volume filtration or by diluting the sample with filtered seawater. The latter may be important to avoid cell overlap, e.g. of diatom chains, or clumping of aggregated cells, particularly of concentrated net plankton hauls.

As with other filtration-based techniques, the physical loss or damage of cells must be carefully monitored during the preparation procedures. Sloppy filtration techniques, leakage of the filtration funnels, overloading the filter and failure to thoroughly clean the filtration apparatus with detergent between usages, followed by rinsing with ethanol then deionised water between each sample, can cause cell loss. Sometimes the sample may leak outside of the grease chalk circle and therefore may not be counted; for this reason it is often preferable to eliminate this circle and merely count the entire surface area under the cover slip.

In comparative testing of the FTF method against whole-cell fluorescence *in situ* hybridization (FISH) and the Utermöhl settling chamber methods for counting cultured cells of the marine dinoflagellates *Alexandrium tamarense* and *A. ostenfeldii* (Rafuse 2004), the filtration-based methods yielded a consistently higher coefficient of variation, most of which was attributed to variable cell loss. Yet in a detailed comparison of 17 alternative cell counting methods for *A. fundyense* (Godhe *at al.* 2007), the FTF method exhibited among the lowest standard errors among replicate counts (n = 4 or 5) within the concentration range of 102 to 105 cells L^{-1}. During the filtration step, cells can be lost within the filtration apparatus, i.e. between the funnel and base or via leakage from the interface between the filter holder and the filter. All standard funnel filtration apparatus (Fig. 1), such as the common clamp-systems obtainable from Millipore™, Gelman/Pall™, Sartorius™, etc., are acceptable for this method, including the threaded mounting (Radnoti™-type) systems for attaching the funnel to the filter base. The clamp-type systems are preferable because they are simpler and do not permit the possible loss of cells between the O-rings (Rafuse 2004) that may occur for threaded-type filter systems. Evidence of this cell loss from concentrated samples may be observed by visual inspection of the funnels and other components after the filter is removed. Use of glass versus plastic (polystyrene) funnels does not appear to be critical, but the funnels should be transparent to observe the filtration process. To maximize transfer of all cells to the filter, the filtration should proceed until the filter surface is just dry (i.e. the upper surface is no longer shiny), but not beyond this point, to preserve cell integrity. Dirty apparatus and ultra-slow filtration (<2 mL min^{-1}) can also lead to adherence of cells to the walls of the filtration funnel; such loss can be considerable – >5% of cells under a worst case scenario and often taxonomically biased towards mucilaginous cells or aggregates. In any case, this selective loss from the harvested sample can be verified if necessary by thoroughly washing down the walls of the funnel system with a high-pressure stream of filtered seawater from a squirt bottle and recovering the cells on a fresh filter under gentle vacuum. If few cells are found, loss of cells in the system can be assumed to be minimal.

Breakage of cells upon contact with the filter surface, and especially sucking the filter beyond the dry point under high vacuum can also account for considerable cell loss or distortion in all filter-based methods. For this reason, vacuum pressure should be kept low (<15 mm Hg) but substantial enough to ensure a steady flow of filtrate (a couple of drops per second or about 50 mL min^{-1} is acceptable). Correct operation of the filtration protocol can be verified by observation under a dissecting microscope of random or haphazard distribution cells on the upper filter surface of unialgal cultures. For mixed algal assemblages, evidence of clumping or patchy cell distribution over the filter surface is a sign of poor filtration technique.

The foregoing are generic strengths and weaknesses of all filtration-based counting methods. Surprisingly, two elements that are specific to the FTF method – freeze-thaw and filter-transfer – do not contribute in a major way to cell loss or morphological distortions when the method is applied correctly. Quick freezing is preferable to slower methods since the former leads to less crystal formation and hence less cell breakage. Cells may also break when the filter is ripped off the frozen sample that contains the cells, or if the cells are weak, not completely frozen, or have thawed too quickly. Practice will generally ensure excellent reproducibility.

Acknowledgements
The authors are indebted to J.C. Smith and K. Pauley, Gulf Fisheries Centre, Dept. of Fisheries and Oceans, Canada for collating details of the method in the unpublished field and laboratory manual for the collection, identification and enumeration of toxic marine phytoplankton from southern and eastern regions of the Gulf of St. Lawrence.

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8 Imaging flow cytometry for quantitative phytoplankton analysis — FlowCAM

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Introduction

In water quality analysis and monitoring the ability to count, capture and save images of the particles or organisms in a sample is advantageous. Traditional "particle counting" methods, such as microscope particle analysis, can be slow and tedious. The FlowCAM, an imaging flow cytometer developed by Fluid Imaging Technologies, captures digital images of particles in a fluid stream using laser light detection, enabling the measurement of many cell parameters, such as length, width, equivalent spherical diameter and fluorescence (Sieracki *et al.* 1998). The captured images from a sample can be studied visually as well as post-processed by a computer to automatically search for particles of a certain type or class. The FlowCAM detects and measures fluorescence emissions at two different wavelengths, typically red and orange fluorescence, indicating the presence of chlorophyll or phycoerythrin within the individual particles/cells in a field sample. This technology is very similar to a flow cytometer that captures only fluorescence and scatter properties of a particle. However, the FlowCAM merges the technologies of a flow cytometer and an imaging microscope – resulting in the Flow Cytometer and Microscope (FlowCAM – Fig. 1). The FlowCAM automatically counts and images particles or cells in a discrete sample. Using the image analysis data generated during sample processing, the FlowCAM software uses image libraries previously created by the user of target groups or classes that can assist in analysis and classification. Overall, the FlowCAM can be customised to accommodate most field environments and can be used to detect and quantify different algal groups within a sample (Zarauz *et al.* 2009), including harmful algal species (Buskey and Hyatt 2006).

Materials

Equipment

For quantitative microphytoplankton analysis (20-300 µm) a Benchtop or Portable FlowCAM (VS IV) is required (Figs 2 & 3). The FlowCAM must be equipped with either a blue (488 nm) or green laser (532 nm) for fluorescent and particle detection. In addition the instrument should have and be setup with, a 4X or 10X objective depending on the size of the cells to be visualised and quantified. Each objective must use a corresponding rectangular. tubular glass Flow Cell (100 or 300 µm depth) in which all cells are to be analysed. For discrete volume sampling a funnel stand or pipet tip apparatus must be used, as provided with the instrument.

Chemicals and consumables

- Filtered Seawater (FSW)
- Distilled water

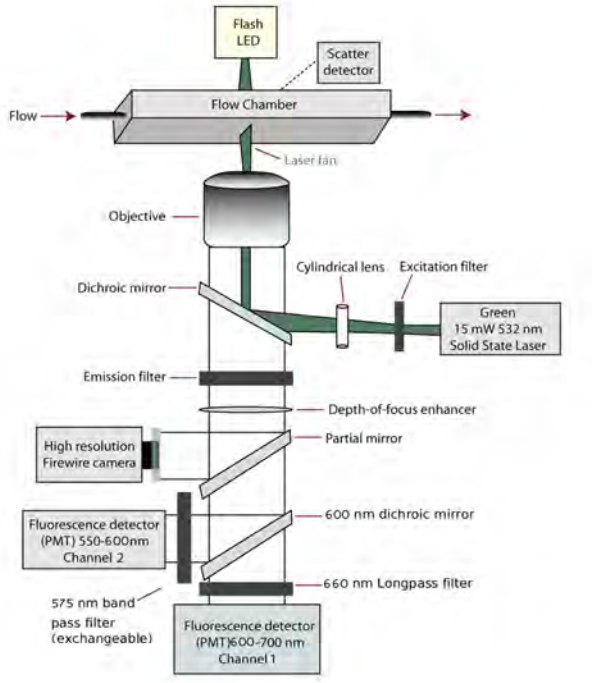


Figure 1. Illustration of the fluidics and optics of the FlowCAM.

- Calibration Beads (5, 10, 20, 50 & 100 µm)
- Disposable plastic pipets tips (1-10 mLs)
- Small funnels
- FlowCAM Flow Cells (100 or 300 µm depth)
- Silicone Tubing
- Nylon mesh (100 or 300 µm)
- Graduated cylinders to measure volumes to be processed

Solutions for preservation

- 10% buffered formalin solution (see Appendix 1 for preparation instructions)
- Formalin: Acetic acid (see Appendix 1)

Methods

The FlowCAM was originally developed for phytoplankton detection and quantification and is ideally suited for analysis of natural field samples that contain specific groups or species of microplankton that are usually counted using traditional microscopic techniques. To begin, the FlowCAM (including the integrated computer) and laser need to be turned on, and the laser allowed to warm up for approximately 20 minutes (according to FlowCAM manual). FlowCAMs are equipped with either a green or blue laser; both can be used for algal fluorescence detection. As the FlowCAM laser is warming

The fundamentals of

The FlowCAM - maging flow cytometer method

Scope
The FlowCAM method captures digital images of particles and/or cells. This enables the instrument to measure cell abundance and many cell parameters, such as length, width, equivalent spherical diameter and fluorescence.

Detection range
The detection range depends on the volume of sample analysed.

Advantages
Manual labour required for processing and handling is minimised, compared to traditional microscopy techniques. The method produces a non-biased digital record and instantaneous image analysis data on every particle or cell within a sample. The captured images from a sample can be studied visually as well as post-processed by a computer to search and quantify cells of a certain type or class. Portable FlowCAMs are also available for field measurements.

Drawbacks
Cells are required to have a distinct morphology to be readily identified by FlowCAM. Different objectives are needed for different phytoplankton size ranges. Preservation of the sample is not recommended, live samples are optimal, as cell autofluorescence can decline or is removed with certain preservation techniques (ie. Utermohls). Heavy particle loads (riverine or estuaries) could interfere with image capture – dilution of the sample for accurate abundances may be required.

Type of training needed
1. To use FlowCAM: Approximately one-two days of training would be required with guidance from a trained individual and follow-up support.
2. To troubleshoot and QC: A more experienced analyst with up to 6 months experience would be more effective at troubleshooting the instrument. The company that manufactures the FlowCAM, Fluid Imaging Technologies, provides on-site, web-based, and over the phone customer assistance when needed.
3. Taxonomic expertise is required to interpret images and results.

Essential Equipment
FlowCAM VS IV with 4X & 10X objectives, associated VisualSpreadsheet (ViSp) software, and Flow Cells.

Equipment cost*
$75,000-85,000 US (depends on the model – Benchtop or Portable), see Appendix 2.

Consumables, cost per sample**
1-5 US dollars.

Processing time per sample before analysis
Ranges from 15 minutes-1 hour depending on the volume run and particle density.

Analysis time per sample
Ranges from 1 hour to minutes. The time consuming part is identifying cells and developing image libraries for automated sample analysis (hours of time upfront).

Sample throughput per person per day
4-36 depending on the time of run and analysis time.

No. of samples processed in parallel
One sample at at time.

Health and Safety issues
Minimal, caution and general safety practices must be used when using the laser within the instrument and preserved samples.

*service contracts not included
**salaries not included

Figure 2. Benchtop FlowCAM.

Figure 3. Portable FlowCAM (VS IV).

up, the FlowCAM VisualSpreadsheet (ViSp) software can be started.

Before running natural field samples, the FlowCAM needs to be ”setup”, meaning the values for triggering using fluorescent detection (thresholds) and the cell size range of particles to be collected need to be determined. The FlowCAM also needs to be properly focused, similar to how a microscope would work. In order to get good visualisation of the particles in each sample optimal focus is required. The set up process usually takes between 20-40 minutes. Once the settings for microplankton detection are determined the settings do not change between samples, unless the ecosystem or background within a sample (such as particle load) changes. More details on how to set up the instrument can be found in the FlowCAM Operators Manual (Anonymous 2009).

For analysing and counting microplankton in natural field samples 4X, 10X or 20X objective are usually used in combination with an appropriate Flow Cell (Table 1). The Flow Cell is a rectangular glass tube that can vary in size depending on the objective used. Silicone tubing is affixed to both ends and allows the sample to pass through the tube to a peristaltic pump downstream of the Flow Cell. The Flow Cell mimics the glass side of a microscope. Note that the Flow Cell is oriented vertically in the instrument differing from the microscope mounted slide that is positioned horizontally. Installation of a 100 µm depth Flow Cell is described in Fig. 4.

Table 1. Recommended cell size range (diameter) and objectives for different FlowCAM Flow Cells.

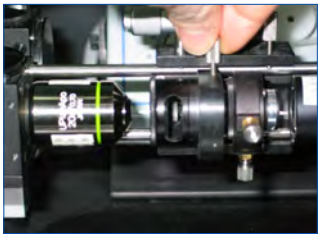
Cell Size Range	Objective	Flow Cell	Flow Cell Dimensions (Depth x Width)
10 µm – 100 µm	10 X	FC100	100 µm x 2 mm
20 µm – 300 µm	4 X	FC300	300 µm x 3 mm
60 µm – 600 µm	4 X	FC600	600 µm x 6 mm

Data Acquisition

1 Each field sample needs to be thoroughly mixed by gentle rotation to resuspend any particles that may have settled;
2 Using a graduated cylinder, a predetermined volume of each sample is dispensed into a funnel or pipet tip;
3 Set the ViSp FlowCAM software to start analysis and collect the data (file folder name and location) in Trigger mode;
4 Start the peristaltic pump, once data acquisition in the ViSp FlowCAM software begins the sample will now be analysed for fluorescent events (phytoplankton) that pass by the laser that is aligned with the Flow Cell.

For most field samples, between 10-100 mL of each sample is analysed and processed. Sample processing rate (flow rate) will depend on the density of cells in the field sample, and the dimensions of the Flow Cell used. As a starting point, set the standard VWR pump to fast mode and the speed dial to 5. As the sample is flowing through the Flow Cell, check the fluorescence trigger rate using ”Trigger Mode Setup”. If the images are appearing at a rate more than one per second begin to turn down the flowrate of the pump until the optimum level is attained. In somes cases the microphytoplankton concentration will be low and there will appear to be no trigger events. The user should be aware that increasing the flowrate too much (PRIME mode) may cause image distortion and prevent accurate image capture. To test the fluorescence settings (thresholds) of the FlowCAM it is recommended that a diluted phytoplankton culture (100-1000 fold) be used initially to verify the settings being used. It is essential that the Flow Cell and funnel are rinsed throughly afterwards to prevent contamination of subsequent samples to be quantified and analysed. Typically, a field sample can be processed in 30-40 minutes.

During the run, the operator should occasionally mix the sample in the funnel using a pipette to prevent particles or cells from settling. The operator should also check the Flow Cell for any potential clogs or blockages. If clogging occurs, the user would typically observe particles sitting at the entrance to the glass tubing. If a clog occurs the operator should end the run and restart the analysis. To prevent clogging from occurring, screening or sieving the sample prior to analysis is recommended using nylon mesh (100 or 300 µm) depending on the depth of the Flow Cell being used. During a run the operator should also periodically pinch the tubing down stream of the Flow Cell in order to prevent large particles from blocking the entrance to the Flow Cell. Near the end of a run the funnel should be rinsed with FSW to ensure all particles are removed from the funnel (and have entered the



Step 1. Remove the CH300 Flow Cell Holder from the Focusing Collar by loosening the thumb screw and pulling the Flow Cell Holder away from the focusing rails. Loosening the thumb screw is accomplished by turning the screw counter-clockwise. The 10X objective can be replaced with a 4X objective.

Step 2. Unscrew the retaining cap from the Flow Cell Holder. This is accomplished by turning the cap counterclockwise until it separates from the Flow Cell Holder.

Step 3. For some FlowCAM models the CH300 Flow Cell Holder has a backer ring (previously installed) that needs to be adjusted when using the 100 µm depth flow cell. The backer ring can be moved by crossing your index and middle fingers and inserting them into the inside diameter of the ring. Move the ring in or out until it backs the flow cell achieving a good fit when the Flow Cell Holder cap is tightened.

Step 4. Place a 100 µm Flow Cell into the pre-cut notches of the Flow Cell Holder. Ensure that the inlet tube is at the same end as the thumb screw. The silicone tubing has been previously attached to the Flow Cell.

Step 5. Reinstall the retaining cap onto the Flow Cell Holder by turning the cap counter-clockwise. The cap is significantly tightened when the Flow Cell cannot be moved. The user will have to hold the Flow Cell straight as cap tightening will tend to twist the Flow Cell. Caution – overtightening will break the 100 µm Flow Cell.

Step 6. Re-install the Flow Cell Holder onto the Focusing Collar and tighten the thumb screw. Connect the inlet tube of the Flow Cell to the sampling funnel using the funnel stand apparatus.

Step 7. Connect the outlet tubing to the fluid pump (peristaltic pump). Ensure the effluent tube is connected to an appropriate waste container. Once the 100 µm Flow Cell has been installed the Flow Cell needs to be thoroughly rinsed with filtered seawater (FSW) and the fluid level brought to the neck of the funnel.

Figure 4. Installation of a 100 µm depth Flow Cell.

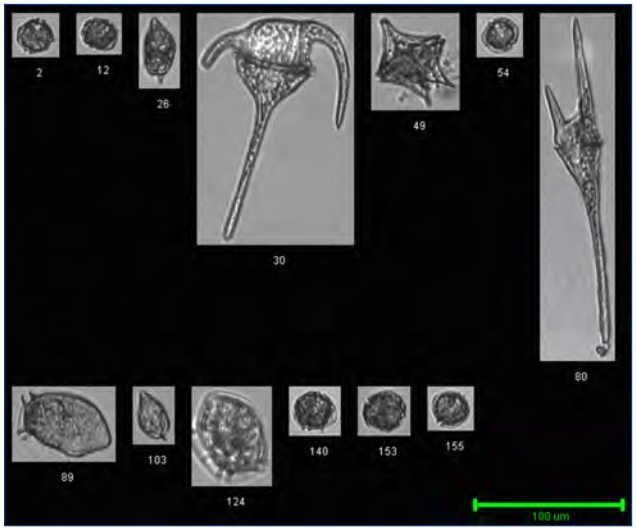


Figure 5. Representative black and white FlowCAM images from a field sample containing the following genera, *Dinophysis, Alexandrium, Protocentrum* and *Ceratium*.

Flow Cell) before it runs dry. Preventing air from entering the Flow Cell between samples will prevent tiny bubbles from forming that could be detected as particles and imaged in the next sample. Any clogs can be removed by reversing the pump direction or inverting the Flow Cell and rinsing with FSW or distilled water.

Data Analysis

Each fluorescent particle/cell is digitally acquired and archived by the FlowCAM ViSp software (Fig. 5). Analysis of the samples is done either immediately upon completion of the sample acquisition or at a later time after many samples have been acquired or archived. The ViSp software allows the operator to use previously acquired image libraries of target species or organisms to assist in sample analysis. The libraries are user generated and can be created from images obtained from cultured organisms or from positively identified natural field images of the target organism. The libraries allow the operator to pattern match each field sample by filtering and sorting the data into user defined catagories. This procedure can be repeated for different target organisms within a single sample or multiple samples. Once the analysis is completed, the positively identified images are verified by the operator, and a total count of each class or group is determined. The final cell concentration of each class is determined using an equation that includes the cell count, volume analysed and Flow Cell Factor – see Formula for Calculating Results below.

Calibration

Prior to microphytoplankton sample analysis, the FlowCAM needs to be properly calibrated using an optical micrometer. The micrometer is used to measure the field-of-view of the camera for each magnification objective (4x & 10x), enabling an accurate size calibration for each pixel. This "calibration factor" becomes part of the context settings for each magnification. Prior to shipment, a new FlowCAM is calibrated to assure accurate measurements of particle concentration (particles/mL) and for sizing particles. Although no in-field calibration is required, a FlowCAM user can analyse polystyrene or latex beads (of known size) for calibration assurance. Calibration beads can be purchased at a variety of commercial companies (10-100 µm, Duke Scientific, USA). To verify the FlowCAM calibration refer to the FlowCAM Operators Manual for a detailed step by step protocol.

Preservation and storage

Unpreserved samples are ideal for quantifying and analysing microphytoplankton in the field, and is recommended. However, this is not always appropriate. Preservation of samples should be done using a 10% buffered formalin solution or formalin:acetic acid, such that the final concentration is approximately 1-2%. Preserved samples should be processed as soon as possible, as fluorescence detection is essential for accurate cell detection and enumeration. Chlorophyll fluorescence within the cell decreases with prolonged storage. Preserved samples are best analyzed using Auto Image Mode, to assure accurate phytoplankton counts. However, more processing time is required in this mode in order to analyze an adequate sample volume. If longer preservation is required, the effects of the storage length should be tested on the FlowCAM prior to processing samples on a routine basis.

Formulas for calculating results

To determine the concentration of the target microplankton group or species within a sample three values are needed:

1 The volume processed by the FlowCAM;
2 The cell count of the target group (based on classification) (N);
3 The Flow Cell Factor.

$$Cell\ concentration\ (cells\ mL^{-1}) = \left(\frac{Cell\ count\ (N)}{Volume\ of\ sample\ (mL)}\right) * Flow\ Cell\ Factor$$

Since the camera on the FlowCAM can only visualise a portion of the Flow Cell (usually between 33-95% - depending on the objective and Flow Cell used) the Flow Cell Factor takes into account the portion of the field of view that is not visualised by the FlowCAM. The Flow Cell Factor will vary depending on the objective/Flow Cell combination utilised. The Flow Cell Factor is calculated automatically by the ViSp software once the correct dimensions of the Flow Cell used are provided by the user. Note that Fluid Imaging Technologies has recently developed a new Flow Cell design that eliminates the need for Flow Cell Factor.

Discussion

The ability to automate the detection and counting of microphytoplankton in field samples is a huge advantage for monitoring aquatic systems. With the development of more automated techniques, such as the FlowCAM there is a movement to employing more remote automated monitoring techniques. The FlowCAM, with further development, could assist in water quality monitoring for both marine and freshwater environments. The FlowCAM is ideally suited for quantifying microphytoplankton between 20-300 µm in size (however, smaller and larger particles can also be detected). Cell classification using the FlowCAM ViSp software is best used on species/groups of phytoplankton that have unique cell characteristics, such as cell size, shape or colour. For ex-

ample, the genera *Dinophysis, Ceratium,* and *Chaetoceros* have many distinctive morphological features which are easier to classify than different species within the genus *Alexandrium*. The FlowCAM is best used to examine a wide range of species within one sample. It can be used as a monitoring tool for coastal projects or in the laboratory, processing discrete samples when needed. In order to best evaluate the instrument a list of Advantages and Disadvantages of the FlowCAM method are provided below:

Advantages of the FlowCAM Method

1 The amount of manual labour required for sample processing and handling is greatly reduced when using a FlowCAM for microphytoplankton analysis. For discrete sample analysis (as described in this chapter) the operator is required to set up and begin a run, but will only occasionally monitor the FlowCAM when it is running and detecting particles;

2 The FlowCAM records a "non-biased" digital record of every particle/cell within a specific size range (determined by the operator) for further analysis using the FlowCAM ViSp software. Using traditional microscope techniques, the operator scans the slide and either seeks out the particles/cells of interest or identifies all the observed cells on the slide. This may result in a biased analysis and count of a sample (which depends on the operator's attention to detail and identification knowledge). The data generated by the FlowCAM is archived and can be reanalysed by more skilled individuals when problems arise or when different analyses are required;

3 In addition to capturing an image for each particle detected by the FlowCAM, the software provides instant image analysis on each particle , up to 30 different image parameters. For example, particle length, width, equivalent spherical diameter (ESD), area-based spherical diameter (ABD), fluorescence, time of flight and aspect ratio are some of the the primary data measurements obtained. Given the data provided, the operator can develop specific algorithms or use previously determined algorithms from the literature for values of interest such as bio-volume and Carbon:Chlorophyll ratio depending on the needs of the application or project;

4 The FlowCAM is portable. Even the benchtop model has a relatively small foot-print (100 x 70 cm) and can be used in the lab or at sea on board ships. The image capture system prevents problems usually associated with vibration;

5 The FlowCAM allows for the visualisation and/or detection of a wide particle size range (1 µm – 1 mm equivalent spherical diameter). To detect across this large size range a series of objectives and flow cells would need to be used. Based on the size of the target organism to be detected (for example, *Alexandrium*), a 10 X objective with a 100 µm depth flow cell would be used. To examine smaller or larger particles within a sample, other objectives and flow cell combinations may need to be used.

Disadvantages and Drawbacks

1 In terms of cell identification it is essential to achieve the best possible focus, otherwise the images will be blurry and will be difficult to analyse;

2 Depending on the ecosystem that is being analysed the phytoplankton cell size range may vary greatly. Each objective and Flow Cell size that can be used has a minimum and maximum cell size range that's possible (similar to the limitations of microscopy). Therefore, to best identify all phytoplankton within a given sample, two FlowCAM runs may be required at two different magnifications. This depends on what magnification is acceptable for cell identification. If cells are too large to pass through the Flow Cell clogging may occur. Therefore, method development at the beginning of a particular project is important. However, if only one particle/cell size is required and cell identification is not necessary– one FlowCAM run may be sufficent;

3 A limited number of preservation techniques for phytoplankton detection using fluorescent based triggering can be used. To assure accurate cell concentrations using preserved samples Auto Image Mode is recommended, but more processing time is required (1-2 hours). The best method of preservation is 1-2% final concentration formalin. Preserved samples should be stored cold and in the dark. Prior to processing let the samples acclimate to room temperature. Lugol's iodine or other iodine based preservation methods are not recommended for use with the FlowCAM as the fluorescence of the particles is lost and the high contrast of the images makes it difficult to identify different phytoplankton groups/genera;

4 In situations where cold samples are being analysed in high humid environments (for example, samples of deep water being analysed on board ships), condensation on the flow cell may interfere with particle detection. The solution to this problem is to allow the sample to attain room temperature prior to analysis. The sample should be preserved prior to manipulation;

5 When the particle load is very high as in riverine samples containing high concentrations of detritus, more than one particle may be captured in a single field of view on the camera using Trigger Mode. Although each particle will have different image analysis values, such as particle length, width, ESD etc., the fluorescent value of both particles will be the same. It is also possible to capture "non-fluorescent" material if the particle load (sediment) is too high and the instrument is detecting particles in fluorescent detection mode. In these cases, Auto Image Mode could be utilised;

6 The operator or user should have some knowledge of phytoplankton identification for analysis of the FlowCAM data.

Type of training required to operate a FlowCAM

1 To perform sample analysis: Approximately one-two days of training would be required with guidance from a trained individual and follow-up support;
2 To troubleshoot and QC: A more experienced analyst with up to 6 months experience would be more effective at troubleshooting the instrument. The company that manufactures the FlowCAM, Fluid Imaging Technologies provides on-site, web-based, and over the phone customer assistance when needed.

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Appendix 1

Preparation of a Buffered Paraformaldehyde/Formalin Solution

Materials
Paraformaldehyde powder
1N NaOH
Stirring/hot plate
Chemical fume hood
pH meter
Phosphate buffer ed saline (PBS) or filtered seawater
Distilled H_2O
G/F filters

1 Mix 900 mL distilled water and 100 g paraformaldehyde powder;
2 Set up on a stirring/hot plate under hood. Heat to approximately 60°C. Do not boil;
3 Stir for approx 1 hour. Turn off heat;
4 Add 100 µL 1N NaOH to “clear” solution. Cool to room temperature. Note: In some cases, not all the paraformaldehyde will go into solution;
5 Add 100 mL phosphate buffered solution or filtered seawater. , depending on whether the samples are freshwater or marine;
6 Filter through GF/F filter to remove precipitate;
7 Test pH. Should be 7.4 - 8.0 (approx. equal to seawater);
8 If necessary, add more NaOH.

This yields a 10% solution (approximately).

Preparation of a Formalin: Acetic Acid Solution

Materials
Formaldehyde (37%)
Concentrated acetic acid

1 Mix equal parts of formaldehyde and acetic acid;
2 Using this solution, add 2.5 mL per 100 mL of sample.

Appendix 2

Table 1. Equipment and costs..

Equipment	**Supplier**	**Cat. Number**	**€**	**US $**
FlowCAM VS IV	Fluid Imaging Technologies (FIT), USA	VS IV or portable VS IV	53,760	80,000
50um Flow Cell	FIT, USA	FC50	28	41
100um Flow Cell	FIT, USA	FC100	19	28
300um Flow Cell	FIT, USA	FC 300	19	28
Nylon Mesh	Wildco, USA www.wildco.com	24-C27 (53 µm) 24-C34 (100 µm) 24-C48 (300 µm)	89	132
Silicone Tubing (1.6 mm ID)	Cole Palmer, USA	K-06411-62	12	18
Calibration Beads	Duke Scientific, USA www.dukesci.com	4205A (5 µm diameter) 4210A (10 µm diameter) 4220A (20 µm diameter) 4250A (50 µm diameter) 4310A (100 µm diameter)	623	927
Plastic Pipets	VWR, USA	14670-103	36	53
Mini-funnel	Hutzler Manufacturing www.usplastic.com	801	0.67	1
Paraformaldehyde	VWR, USA	MK262159	38	56
Sum approx.			**54,536**	**81,284**

9 Detecting intact algal cells with whole cell hybridisation assays

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Introduction

Fluorescence in situ Hybridisation (FISH)

Ribosomes are the sites for protein synthesis in all cells. All cells are packed with many ribosomes because protein synthesis is an on-going cellular process. Each ribosome is composed of ribosomal RNAs (rRNAs) and accompanying proteins. The RNAs within the ribosome fold into a shape that permits the synthesis of the proteins and this folding of the molecule is maintained for proper functioning of the ribosome. If mutations occur that violate the folding of the molecule, then the molecule is non-functional. Within the interior of the folded RNA is the conserved sequence regions. These cannot change otherwise the molecule will not fold properly. The more variable regions of the RNA molecule are found on the surface of the molecule which do not interfere with the folding of the molecule. Thus, based upon conserved and variable regions of the rRNA, signature base sequences of varying taxonomic specificity can be found (Fig. 1). In other words, regions can be identified in the rRNAs that will recognise all members from as broad a group as a kingdom of organisms or be so selective to identify only a species or cluster of strains in that species. These short sequences have been used to develop probes for the identification of organisms at various taxonomic levels. Given the vast amount of rapidly accumulating sequence data for all kinds of organisms, it is now possible to develop these probes for a broad spectrum of taxa. When these probes are coupled with a fluorescent marker, the target organism can be easily identified by a technique known as Fluorescence *in situ* Hybridisation (FISH). Fluorescence *in situ* hybridisation enables the rapid detection of different species or strains (Amann 1995). This technique has been successfully applied for the detection of harmful algae (Anderson 1995, Miller and Scholin 1996, 2000, John *et al.* 2003, Anderson *et al.* 2005) as well as algal classes (Simon *et al.* 1997, 2000, Rhodes *et al.* 2004a, 2004b, Eller *et al.* 2007) and other taxonomic hierarchies (Groben *et al.* 2004, Groben and Medlin 2005, Töbe *et al.* 2006).

Basic Principles of FISH

The target algal cells are hybridised with fluorescently (e.g. the fluorochrome fluorescein isothiocyanate; FITC) labelled oligonucleotide probes, which bind to the complementary target sequence of the rRNA in the ribosomes (Fig. 2). This results in a bright labelling of the entire algal cell because of the high target number of ribosomes in cells (Figs. 2-4). A list of all algal probes can be found on the EU PICODIV (Diversity of Picoeukaryotic Organisms) website (http://www.sb-roscoff.fr/Phyto/PICODIV/). In each RNA molecule the more conserved positions can be used to develop taxonomic probes e.g. on a class level, whereas the variable regions are used for lower taxonomic levels.

Initially, the cells are fixed with a preservative that actually makes the cell membrane permeable for the entry of the probe into the cell. The fluorescently labelled probe finds its way to the ribosome and binds to the region of the rRNA to which it is complementary, forming a duplex. When the sample is viewed under a fluorescent microscope using light of the correct wavelength, the fluorochrome is excited and the cell of interest can easily be visualised. The three methods described in this chapter all incorporate these basic principles of FISH; however, there are slight variations in their protocols

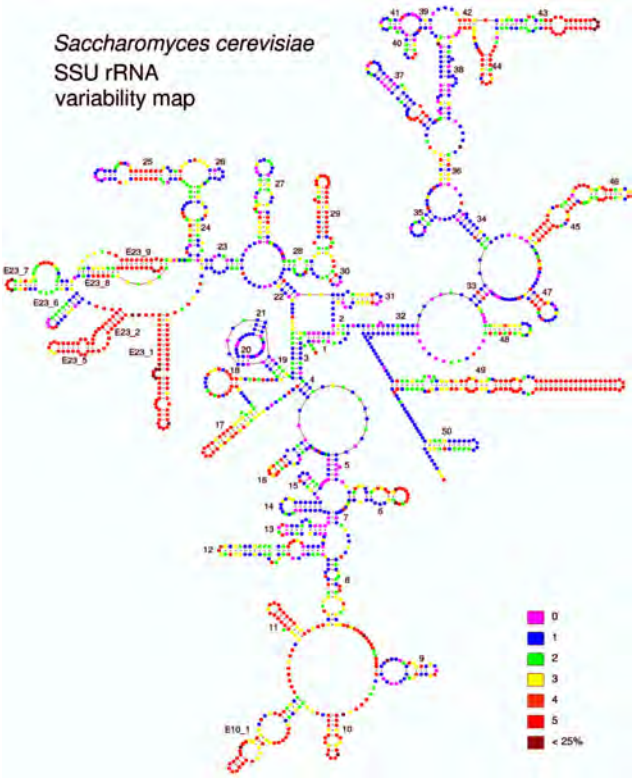

Figure 1. Variability map of the 18S rRNA molecule. Source: Bioinformatics.psb.ugent.be.

The fundamentals of

The fluorescent *in situ* hybridisation (FISH) method

Scope
Detection and quantification of target phytoplankton species.

Detection range
Detection of microalgal RNA by FISH is very sensitive. The number of cells that can be detected depend on the sample volume. High biomass can obscure the view of target cells.

Advantages
Relatively inexpensive if appropriate fluorescence micrsocopy facilities are available. Visible observation of cells is possible. Simple and easy to use. Sample volumes can easily be adjusted. Simultaneous labeling and detection of multiple species is possible.

Drawbacks
Probes are only available for a limited number of target species. Rigorous optimisation and specificity testing on local strains is required before the method can be implemented. Finite storage time for samples. Processing procedure may result in cell loss. Relatively expensive start-up costs. Intensity of the positive reaction may vary with cell conditions. Access to molecular expertise is essential. Appropriate laboratory facilities for storage and processing of probes and reagents are necessary.

Type of training needed
Instruction in setting up this technique should come from a person with an in-depth knowledge and experience of molecular biology. Approximately one week of supervised training required to properly perform the method.

Essential Equipment
Epifluorescence microscope, filtration unit, hybridisation oven, various air displacement pipettes, vacuum pump.

Equipment cost*
Total set-up cost = 20000 € (30000 US $)
See Appendix, Table 1 for details.

Consumables, cost per sample**
Cost per sample = 1.67 € (2.50 US $). See Appendix, Table 2 for details.

Processing time per sample before analysis
In total ca. 4 hours per sample. This excludes fixation time which is 1-24 hours.

Analysis time per sample
Microscopical analysis 45 minutes to 2.5 hours/sample.

Sample throughput per person per day
A trained person can process and count 16 or more samples/day.

No. of samples processed in parallel
Number of samples processed in parallel: 14-24 (dependent upon manifold capacity).

Health and Safety issues
Formamide, formalin, methanol and DAPI are hazardous chemicals. Proper safety guidelines should be employed when using them - lab coat, eye protection and gloves are required. Disposal of all waste products should follow prescribed laboratory procedures. Continual microscope work could result in strain injury. It is important to incorporate breaks into the daily analyses time. Ergonomic adjustments to the microscope and the working place is recommended.

*service contracts not included
**salaries not included

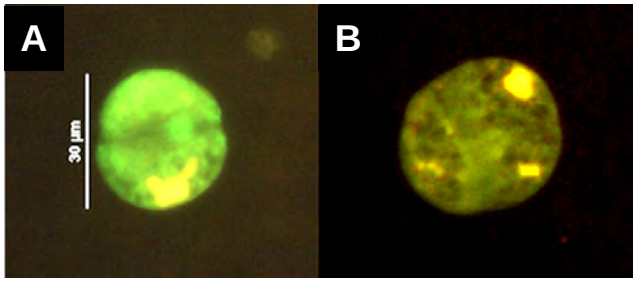

Figure 2. Micrographs of *Alexandrium fundyense* cells using the FISH method: A FISH with FITC-labelled probe NA1 (Miller and Scholin 1998); B Negative control, no probe. 400X.

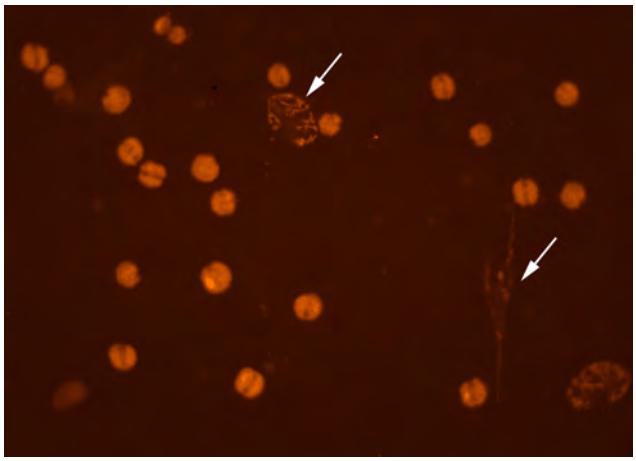

Figure 3. Fluorescence *in situ* hybridisation with *Alexandrium fundyense* cells with Cy3-labelled probe NA1. Note the autofluorescence of the dinoflagellate cells of the genera *Dinophysis* and *Ceratium* in the background (arrows) 200X.

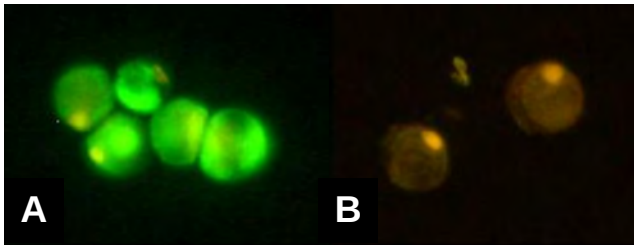

Figure 4. *In-Stu* Hybridisation with *Alexandrium ostenfeldii* cells: A FISH with FITC-labelled probe Aost (John *et al.* 2003). B Negative control, no probe. 400X. Cawthron laboratory.

that offer the user various advantages depending upon their specific sample requirements. Optimisation of the FISH assay is required for each new probe/probe set and should involve testing a matrix of conditions including:

- Hybridisation buffer - 1X, 5X, respectively, depending on the protocol used. Set buffer and if required, 5% intervals of formamide concentrations. See Appendix, Table 3 for more details on how to make up the different buffers
- Hybridisation length - longer hybridisation times can result in non-specific binding
- Hybridisation temperature - low temperatures can cause non-specific binding and temperatures that are too high may inhibit binding
- Hybridisation wash buffer and wash duration – these steps influence the intensity of the target label as well the degree of non-specific labelling

The strongest positive signal from the target organism and lowest cross reactivity from non-target organisms will define the optimal assay conditions for the probe.

Materials

Laboratory Facilities

This method can be used in laboratories and on board research vessels.

Required Equipment (essential)

The whole cell method requires the following equipment:

- Epifluorescence microscope
- Filtration manifold
- Hybridisation oven or water bath with thermometer
- Air displacement pipettes (10 µL – 10 mL)

Equipment, Chemicals and Consumables

The equipment, chemicals and consumables used in this method are presented in the Appendix, Tabels 1-2 at the end of this chapter. Suppliers, catalogue numbers and estimated cost in Euros and US Dollars for the year 2007 are also listed in Appendix.

Method

Three different fluorescence *in situ* hybridisation (FISH) methods are presented. Method 1 and 3 use the LSU probe, NA1, recognising the North American *Alexandrium* ribotypes (*Alexandrium fundyense /catenella/tamarense*) 5'-Fluor/AGTGCAACACTCCCACCA -3' (Anderson *et al.* 1999). In method 2 another LSU probe, NA2, which also discriminates North American ribotypes was used: 5'-Fluor/ AACACTCCCACCAAGCAA -3' (John *et al.* 2003). This probe has a 5 bp (base pair) shift of NA1 sequence to avoid the hair pin loop that this probe makes with itself that might reduce its hybridisation ability at low temperature.

Methods 1-3 below are variations of the FISH assay using *Alexandrium fundyense* as the target organism. Each method differs slightly according to different personal preferences with regards to some of the equipment used, preservation methods and the hybridisation protocol (see Table 1 for more details).

With the appropriate filtration set, many samples can be processed in parallel. Samples in methods 1 and 2 can be easily filtered overnight for processing in the morning with probe hybridisation. It is preferable that no significant delays occur during the sample processing steps when using method 3. With any of the methods, the hybridisation takes approximately 2 hours and the filters can be enumerated thereafter. The number of samples to be processed and counted will vary with each worker; the majority of time is devoted to microscopic examination of the sample. The time spent on each sample varies depending upon the target cell density, the concentration of non- target cells, detrital matter and counting method employed. The reader is referred to chapter 14 on the laser scanning cytometer to avoid personal examination of the filters.

Table 1. Summary of the differences, advantages and disadvantages between the three methods discussed in this chapter.

	Differences between methods			Advantages	Disadvantages
	Method 1	Method 2	Method 3		
Fixative	Saline ethanol	Saline ethanol	Formaldehyde with methanol extraction	Formaldehyde/methanol allows long term storage of samples (> 1 year @ -20 ºC)	Use of hazardous chemicals and an extra centrifugation step
				Saline ethanol is not hazardous	
Hybridisation buffer	5X or 1X Set Buffer	5X SET Buffer with Formamide	5X SET Buffer with Formamide		Use of an hazardous chemical
Wash buffer	1X Set Buffer	1X Set Buffer	0.2X Set buffer	Method 1 does not require a separate wash solution for dinoflagellates.	
Equipment	Water bath	Hybridisation oven	Hybridisation oven	Water bath is not very expensive A hybridisation oven is easy to use	A water bath is cumbersome to work with A hybridisation oven is more expensive

Method 1

Materials

Solutions for Fixatives

- Modified Saline Ethanol Solution (Miller and Scholin 2000; see Appendix, Table 3 for details).

This fixative is stable at room temperature for several months without precipitate formation. Note: 300 mL of fixative is sufficient for approximately 70 reactions. The modified saline ethanol does not form precipitates and can be made in advance, whereas the unmodified version with a higher ethanol concentration forms precipitates and should be made just before use.

- 25X SET buffer (discard after 12 months; see Appendix, Table 3 for details)

Solutions for Fluorescence *in situ* Hybridisation

The hybridisation buffer concentration needs to be optimised for each probe set (see the discussion in 'Basic Principles of FISH').

- 1X SET buffer (used for dinoflagellates from the genera *Karenia* and *Alexandrium*; see Appendix, Table 3 for details)

or

- 5X SET buffer (used for diatoms from the genus *Pseudo-nitzschia*; see Appendix, Table 3 for details)

- Mix solutions into a baked 500 mL Duran bottle and filter through a 0.45 µm filter. Add 1 mL Polyadenalyic acid (12.5 mg mL^{-1}, Sigma Chemical, P-9403)

- Wash buffer: 1X SET buffer (see Appendix, Table 3 for details) in sterile Milli-Q water

Probes

Probes are received as a dried powder and need to be made up to a concentration of 200 ng μL^{-1} i.e. 50 µg of probe in 250 µL of 1X TE buffer, pH 7.8- 8.0. Make sure all equipment is RNase free and the area is in dim light. Divide into 50 µL aliquots and store at 4 ºC. Probes stored this way will remain stable for 4 months, otherwise, samples need to be lyophilised and stored at -80 ºC for long term storage.

Additional Equipment

- Milleriser Rig (includes tubes, bases, o-rings, lids)
- Water bath with heater and thermometer
- Hand held vacuum pump and trap
- 2-20 µL micropipette + sterile tips
- 100 - 1000 µL micropipette + sterile tips
- 1-10 mL pipette + sterile tips
- Glassware (including Duran bottles) baked for 4 hours at 160 °C
- Latex disposable gloves
- Glass slides
- 22 mm x 22 mm glass cover slips
- Fine tip tweezers
- 500 mL volumetric flasks, acid washed in 3N HCl
- Poretics Polycarbonate filters; 3.0 µm pore size; 13 mm diameter (Osmonics Inc.). Note that 5-8 µm pore size filters can be used for larger cells
- Slowfade® Gold antifade reagent (Molecular Probes, Invitrogen Detection Technologies, S36936)

Fixation

1 Gloves must be worn at all times to avoid contamination of samples with human derived RNAases;
2 Switch water bath heater on, set at 45 ºC;
3 Set up Milleriser Rig (Fig. 5). Assemble one filter set for each species specific probe, plus a positive control, SSU-targeted universally conserved sequence (Embley *et al.* 1992, Field *et al.* 1988), a negative control (the complement of UniC) and a 'no probe' control;
4 Using tweezers, place the o-ring into the tube. Place a filter onto the o-ring ensuring that the shiny side faces the sample. Screw the base into tube and tighten until finger tight. Do not over tighten. Place assembled tube into Milleriser Rig. Attach tube of vacuum trap flask to the Milleriser Rig and tube of vacuum pump to outside flask outlet;
5 Ensure valves are closed (i.e. horizontal);

6 Live field samples: Add 5 mL saline ethanol fixative to each tube. Add live sample to the fixative. Use 5-10 mL of field sample (the volume can be varied according to cell numbers present). Filter down to 3 mL and add a further 3 mL of saline ethanol fixative;
7 Lugol's iodine treated samples: add 5 mL saline ethanol fixative to each tube. Add 10 mL of Lugols treated sample to the saline ethanol fixative. Add 3 drops of 3% Sodium thiosulphate (decolouriser) to each tube using a Pasteur pipette;
8 Cap tubes and tap gently to ensure no air bubbles are sitting on the filter. Let the sample stand for 1 to 24 hours, occasionally tapping gently to remove any air bubbles;
9 Filter fixed samples ensuring the solution level does not drop below the level of the gasket. Do not let pressure gauge read over 10 mgHg. Fixed samples can be kept for up to 4 weeks when stored at 4 ºC.

Fluorescence in situ hybridisation

10 Add 2 mL 1X SET hybridisation buffer to each tube;
11 Filter samples as above - do not let filters dry out;
12 Add 0.5 mL 1X SET hybridisation buffer to each tube;
13 Darken room as much as possible for following steps as probes are light sensitive;
14 Add 12 µL (final concentration in hybridisation 4.8 ng μL^{-1}) probe to tubes. Gently mix the pipette tip in the hybridisation buffer taking care not to touch the filter;
15 Put lids on tubes. Place the Milleriser Rig and tubes in a water bath, ensuring that the solution in the tubes is covered by the water level. Cover water bath with lid (or aluminium foil) to prevent light exposure. Leave in water bath for 1 hour;
16 Remove from water bath and filter as above;
17 Add 2 mL 1X SET hybridisation buffer and leave at room temperature for a few minutes;
18 Gently filter samples until dry;
19 One by one disassemble tubes, remove filters using tweezers and place on labelled slides;
20 Pipette 12 µL of Slowfade® Gold antifade reagent onto each filter and add coverslip;
21 Keep slides covered (i.e. in the dark) until they are ready to be viewed. Colour reaction is enhanced if slides are left to sit in the dark for about 30 minutes before analysing;
22 Analyse slides using an epifluorescence microscope (excitation 490 nm; emission 520 nm) at 200X magnification (Fig. 4);
23 Cells fluoresce brightly if the probe hybridises with target rRNA. This defines a positive result. Species-specific probe filters need to be compared with the positive, negative and 'no probe' controls to discriminate positive fluorescence from autofluorescence exhibited by non-target cells;
24 Results may be recorded as a comment based on the intensity of colour and the pattern of fluorescence. For example: "++" indicates a strong positive result, where cells are very brightly coloured with the flurochrome "+" indicates a positive result or positive cells present. "-" indicates no cells are showing a positive result, cells may have a natural orange autofluorescence;
25 Cells should be counted after an initial examination of each filter. Positive cells on the positive control filter must be counted as well as cells on all the species filters;

Method 2

Materials

Solutions for fixation

Saline ethanol (Scholin *et al.* 1996; see Appendix, Table 3 for details), prepared freshly for each experiment because of the formation of precipitates or modified saline ethanol (Miller and Scholin 2000; see Appendix, Table 3 for details) as described in method one.

Solutions for Fluorescence *in situ* hybridisation

- Hybridisation buffer
- 5X SET buffer (see Appendix, Table 3 for details)
- 0.1 % (v/v) Nonidet-P40
- x % (v/v) Formamide*

*Note: Formamide concentrations must be determined for every single probe by performing FISH assays with formamide concentrations in 5 % intervals and microscopically verified. The appropriate formamide concentration will brightly label all of the target cells tested with no cross hybridisation. The formamide addition will reduce the binding temperature of the probe so that it can hybridise specifically at 50 ºC. The hybridisation buffer should be filter sterilised if stored for a longer time.

Wash buffer

- 1X SET buffer (see Appendix, Table 3 for details) in sterile Milli-Q water

Probes

Fluorescently labelled probes purchased from Thermo Scientific, Germany are delivered lyophilised. Stock solution of 1 µg μL^{-1} should be prepared and working solutions of 500 μL^{-1} and 50 ng μL^{-1} in 1X TE buffer, pH 7.8- 8.0. Probe stock solution should be stored at -80°C and working solutions at -20°C.

Additional Equipment

- Filter vacuum manifold (Millipore, Bedford, USA) or glass filter equipment
- Hybridisation oven
- Vacuum pump
- Pipettes 1-20 µL, 100-100 µL + sterile tips
- 1-50 mL pipette + sterile tips
- Autoclaved glassware
- Disposable gloves
- Glass slides
- Coverslips
- Tweezer
- White polycarbonate filter membranes: 47 mm or 25 mm diameter, pore size depending on cell size (Millipore, Bedford, USA)
- DAPI (4'-6-Diamidino-2-phenylindole, Invitrogen, Karlsruhe, Germany)
- Citifluor (Citifluor Ltd., Cambridge, UK)
- Colourless nail varnish

Fixation

1 Filter approximately 5 mL sample down onto a polycarbonate filter with the lowest possible vacuum and incubate in the fixative for at least 1 hour at room temperature or overnight at 4 ºC;
2 Incubate the filter for 5 minutes with hybridisation buffer at room temperature to avoid precipitation. Air dry the

filter and use directly for FISH. Alternatively the filter can be stored at room temperature for at least 1 month.

Filters can also be cut in several pieces and treated individually for the detection of additional algal species in one sample. This has to be taken into account when calculating the final cell density.

Fluorescence in situ Hybridisation

3 Apply 60 µL of hybridisation buffer containing the labelled probe onto the filter and hybridise for 2 h in the dark at 50°C. The final probe concentration in the buffer should be 5 ng μL^{-1}. Controls are espeically recommended for newly designed probes. These should include (1) A 'no probe' control: the same procedure without the addition of a probe. (2) A 'positive control' where a known working probe is used. (3) A 'negative hybridisation control' where a 'nonsense' probe is used which would not bind to any of the cells because of its target sequence;

4 Place the slide in a darkened box with moistened filter paper to provide a humid chamber for the hybridisation to take place. The hybridisation temperature is kept at 50°C for all probes as the thermal melting point of the probe is compensated by the addition of formamide to enhance the specificity of each probe. Readers are referred to Amann (1995) and Groben and Medlin (2005) for a full description of how probes should be developed. It is important to ensure that the entire filter piece is covered with liquid and if necessary, more buffer-probe mixture should be used. The fluorescently labelled probe is light sensitive, so the filters should be kept in the dark for the rest of the procedure, i.e. cover them during incubation times and minimise exposure to light when they are handled;

5 Terminate hybridisation by washing the filters in prewarmed (50 °C) 1X SET wash buffer for 10 minutes at 50 °C. After washing, dry the filters by blotting onto Whatman filter paper.

Counterstaining and Validation using Microscopy

6 Mix one mL of Citifluor antifade + 0.5 mL Milli-Q water + 1.5 µL DAPI (1 µg μL^{-1});

7 Place the filters on a glass slide; two filters, 25 mm in diameter, can be placed side by side on one glass slide. Add 60 µL DAPI/Citifluor mixture to the filters, place a cover slip over the filters and seal edges with nail varnish. Store slides in the dark until examined. Slides can be kept frozen for several months without loosing fluorescence signal;

8 Analyse by epifluorescent microscopy using the appropriate filter set for the fluorochrome attached to the probe (Fig. 2). When bound to the rRNA of target cells, positive labelled cells fluoresce bright and are counted as positive signal. Also the intensity of the given fluorescence has to be recorded. The presumed positive results must be compared with positive, negative and 'no probe' controls to differentiate between a real signal from the bound probe and nonspecific binding of probes or autofluoresence.

Method 3

Materials

Solutions for Fixation

- Formaldehyde, 37% (Fisher Scientific, F79P-4)
- 100% ice-cold methanol (Fisher Scientific, A452SK-4)

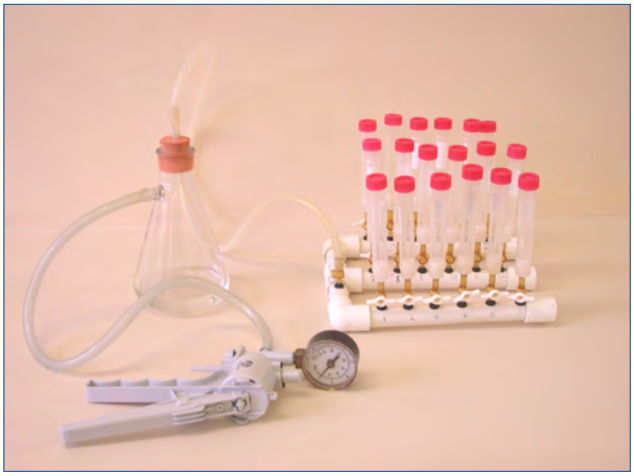

Figure 5. Custom made Milleriser Rig.

Solutions for Fluorescence *in situ* hybridisation

- 25X SET buffer (as described in the Appendix, Table 3)
- 5X SET Hybridisation buffer (to process 14 samples)
  20.4 mL Milli-Q water
  6.0 mL 25X SET buffer
  300 µL Igepal CA-630 (Sigma Chemical, I 3021)
  300 µL Polyadenylic acid (poly A) 10 mg mL L^{-1} (Sigma Chemical, P 9403)
  3.0 mL Super Pure Formamide (Fisher Scientific, BP228-100)

Probes

For the North American *Alexandrium* ribotypes (*Alexandrium fundyense/catenella/tamarense*) the NA1 oligonucleotide probe can be used: 5'-Fluor/ AGTGCAACACTCCCACCA -3' (Anderson *et al.* 1999). Probe stocks should be stored in dry 100 µg aliquots at -80 °C. Resuspend probe stocks in 500 µL 1x TE [TE-Tris-EDTA buffer 100X concentrate (Sigma Chemical, T-9285); working stock 200. Add 360 µL of resuspended probe to 15 mL hybridisation buffer for incubations.

- Wash buffer (0.2X SET)
  120 µL 25X SET buffer
  14.880 mL Milli-Q water

Additional Equipment

- Filter vacuum manifold (Promega Corp., A7231) with custom made 25 mm filter funnels, (modification of the ones described by Scholin *et al.* (1997) and are similar to those in Figure 5)
- Filter membranes: 25 mm Cyclopore membrane (Whatman Inc., 5 mm pore size, 09-930-14E)
- ProLong Gold antifade reagent (Molecular Probes, Invitrogen Detection Technologies, P36930)

Fixation

1 Preserve a 14 mL sample with 0.75 mL formaldehyde (5% v/v, final concentration = 1.9% formaldehyde) in a disposable 15 mL centrifuge tube;

2 The 14 mL volume may be a raw water sample or a concentrated sample generated using filtration;

3 In the field, 2-4 lof water is typically filtered through a 20 µm Nitex sieve (Nitex mesh, Sefar America, Inc., fitted at the end of a 3 in. diameter PVC tube) and the collected

cell material is resuspended to the 14 mL mark and preserved with 5% v/v formalin;

4 Store the samples at 4 ºC for a maximum of 36 hours until they can be centrifuged at 3000 x g, for 5 minutes at room temperature;

5 Aspirate the supernatant and resuspend the cell pellet in 14 mL of ice-cold methanol to extract the chlorophyll and to stabilise the rRNA. Samples are required to stand in methanol for at least 1 hour prior to hybridisation but are stable for many months when stored at -20 ºC.

Fluorescence in situ Hybridisation

6 Add a volume of sample to a filter funnel containing a Cyclopore filter. Filter the sample to near dryness using the lowest possible vacuum;

7 Add 1 mL of pre-hybridisation buffer and incubate for 5 minutes at room temperature. Note: The filter funnel valves should only be open when filtering; they should remain closed at all other times;

8 Filter the pre-hybridisation buffer and add 1 mL hybridisation buffer with added probe. Cap the filter tubes and place the manifold in a black plastic bag. The bag should contain several wet paper towels to provide a humid environment. Seal the bag and place the samples in a dry heat incubator at 50 ºC (±2 ºC) for 1 hour;

9 Complete the hybridisation reaction by filtering the hybridisation buffer plus probe and adding 1 mL prewarmed (50 ºC) 0.2X SET wash buffer. Allow this to incubate for 5 minutes at room temperature;

10 Filter the wash buffer. Keep the filter funnel valve open and continue to apply vacuum to the sample until the filter has been placed onto a microscope slide. Add 25 µL ProLong Gold antifade reagent and a coverslip to the filter. Samples can be stored at 4 ºC in the dark for several weeks prior to observation, but, best results are obtained by viewing the sample immediately after hybridisation;

11 Samples can be observed and counted with epifluorescence microscopy, at 100X using the appropriate filter set. For Cy3, Chroma 41032 or FITC, Chroma 41012 (Chroma Technology Corp.) filter sets are recommended (Fig. 3).

Formulas for Calculating Results

Ideally the entire filter is examined and positive cells enumerated.; hence, the cell number is reflective of amount of original sample that was processed and preserved as well as to the volume of the preserved sample that was placed onto the filter for hybridisation. It should be noted, that only the filter area is counted and not the entire area under the coverslip, which is usually larger than that of the sample filter. If target cell densities are high and warrant only counting a portion of the filter, then a decision has to be made on how to proceed with the count to afford a statistically reliable value. Andersen and Throndsen (2004) provide a good review on this in their chapter in the Manual on Harmful Marine Microalgae.

A generic formula to calculate cells L^{-1} for all 3 methods:

$$Cell\ concentration\ (cells\,L^{-1}) =$$

$$= \left(\frac{Positive\ cell\ count\ on\ whole\ filter\,(N)}{Volume\ of\ sample\ (mL)} \right) * 1000$$

where N is the number of positive cells on the whole filter and V (mL) is the volume of sample used.

To calculate species composition of a bloom

The FISH assay can be used as a secondary test to light microscopy analysis when a designated cell count for a particular genus (e.g. *Pseudo-nitzschia*) has been exceeded and speciation is required to determine risk associated with toxicity. In cases where the cell count has been determined previously for the target genus, the percentage that each species comprises in the sample (i.e. the total species positive counts) can be determined using the following formula:

$$Percentage\ species\ composition =$$

$$\left(\frac{Positive\ species\ cell\ count\ on\ whole\ filter\ (S)}{Positive\ genus\ cell\ count\ on\ whole\ filter\ (T)} \right) * 100$$

where S is the number of positive cells on a species-specific filter and T is the total number of genus positive counts.

Percentages calculated from the FISH data can be applied to the original cell count to get an approximate density of each species in the sample. Pieces of a filter can also be analysed. Counts on the portion of the filter can be calibrated in a similar fashion to those of the Utermöhl method (chapter 2). This removes the requirement for analysing the entire filter, provided that a sufficient proportion of a filter is analysed for statistical significance and that the proportion is the same for each species-specific filter.

Discussion

Whole cell, fluorescently labelled oligonucleotide enumeration-based assays for HAB species can be a simple, effective and efficient tool for counting natural phytoplankton samples. Empirical trials with more traditional counting methods, as discussed in this manual and other studies (e.g. Anderson *et al.* 2005), need to be conducted to determine the specificity of the probe with the target species. This is because not all species-specific probes will label morphologically identical organisms because of their genetic dissimilarities. If a suitable probe has been shown to hybridise with the target organism, and the required equipment to perform the hybridisation and subsequent microscopic analysis are available, then this technique can easily be used to enumerate numerous samples by researchers with limited laboratory and microscope experience. Automated counting systems, such as the ChemScan (Chemunex, France), make this operation much faster (Töbe *et al.* 2006).

The specificity that each probe binds to the target species rRNA needs to be fully evaluated in optimisation and cross reactivity trials. To optimise the FISH hybridization parameters for each probe, assay conditions such as reagent concentrations, hybridisation temperature and time can be manipulated to produce stronger epifluorescent signals. Methods described in this manual (such as light microscopy and Calcofluor staining), and in other studies, (e.g. Anderson *et al.* 2005) must be conducted to determine the extent (if any) of cross reactivity with non-target species.

Careful consideration must be given when determining the assay conditions to balance signal intensity against cross reactivity. Positive, negative and 'no probe' controls are critical for this. Signal intensity of the target species compared with the positive control will give an indication of optimal assay conditions. The negative control will indicate non-specific binding caused by sub-optimal conditions. The 'no probe' control is critical for assessing the amount of autoflourescence exhibited by the cells, which must not be confused as a positive signal.

Consideration also needs to be given to the choice of sample preservation as two of the methods described herein utilise the relatively safe ethanol/SET preservative, but, these do not afford the luxury of long-term sample storage. Formalin fixation followed by methanol extraction and -20 ºC storage allows for long-term sample archiving and subsequent hybridisation, but, involves the use of hazardous chemicals and an extra centrifugation step to remove the formalin seawater supernatant from the cell pellet. Both fixation methods will provide the user with good labelling intensity of the target cells.

In New Zealand, the FISH assay is used as a supporting tool to light microscopy providing additional information to regulators. Multiple species-specific probes can be run as a screen to determine the composition of a bloom when a sample has returned elevated cell counts of potential species of concern. The FISH assay enable rapid identification to the species level in such cases; a task that would be both time consuming and require a significant level of experience to achieve the same results using light microscope techniques.

To increase cell detection limits for field samples, large volumes of water (1-8 l) can be filtered through an appropriate mesh size for concentration purposes, if the target organism is amenable to this type of concentration (e.g. Anderson *et al.* 2005). The captured cell material can then be washed into a centrifuge tube, preserved and an aliquot of the sample slurry can then be processed for the FISH assay. One of the benefits of using FISH is that samples with high biomass as well as difficult to identify species can be easily examined and enumerated by an inexperienced microscopist. A 2 Litre sample of field material can be concentrated down to a volume of 15 mL, of which, 7.5 mL is then processed for FISH affording a limit of detection of 1 cell L^{-1}. One Litre is more than sufficient, unless the study, e.g. picoplankton, calls for greater volumes. The sample volume used in the assay can be adjusted based on cell counts derived from light microscope analysis on test samples.

This method is suitable for all species for which a probe has been designed and are amicable to the preservation methods outlined here. The detection limit of one cell per filter can easily be achieved by manual counts or if automatic counting devices are used, such as the ChemScan solid phase cytometer (see chapter 14 and Töbe *et al.* 2006). The accuracy of manual counting depends on the amount of debris in the sample and the skill of the microscopist to discriminate the positive signal against the weak autofluorescence of other cells. In order to ensure that the method works well, it is important that good temperature control is achieved during the hybridisation step. It is also imperative that the excess probe is washed off the filter in order to reduce background fluorescent interference.

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Appendix

Table 1. Equipment and Suppliers

Equipment	Supplier	Cat. Number	€	US $	Method
Filter Manifold	Millipore	XX2702550	400	587	2
Vacuum pump	Omnilab	9.881	391	574	2,3
Incubator ”Shake’n’Stack”	VWR	7996	2310	3387	2,3
Epifluorescence microscope, Eclipse E800	Nikon	MAA600BA	25000	36.654	2
Epifluorescence microscope, Axioimager A1	Carl Zeiss	4300050000000000	16000	25000	1,3
Milleriser Rig	Custom made		55	80	1
Waterbath	Grant	JBAqua-26	1130	1650	1
Filter Manifold	Promega	A7231	76	114	3
Pipette 2 µL – 20 µL	Fisher Scientific	05-402-87	193	289	1,2,3
Pipette 20 µL – 200 µL	Fisher Scientific	05-402-89	193	289	1,2,3
Pipette 200 µL – 1000 µL	Fisher Scientific	05-402-90	193	289	1,2,3
Pipette 1 mL – 10 mL	Fisher Scientific	05-403-121	193	289	1,2,3

Table 2. Chemicals and Suppliers

Equipment	Supplier	Cat. Number	€	US $	Method
Fixation					
Ethanol, absolute,1l	Merck	1.009.832.500	73	107	1,2,3
Methanol, 100%	Fisher Scientific	A452SK-4	61	90	3
Milli-Q water	Most labs have an in-house supply				1,2,3
Sodium chloride, 1 kg	Sigma-Aldrich	S9888	23	34	1,2,3
Tris/HCl, 500 g	Sigma-Aldrich	T3253	96	141	1,2,3
Nonidet-P40, 100 mL	Roche	11754599	74	109	2
EDTA, 500 g	Sigma-Aldrich	E7889	56	82	1,2,3
Formaldehyde, 37%	Fisher Scientific	F79P-500	27	40	3
Isopore white polycarbonate membrane filter, 3 µm pore size, Qty. 100	Millipore	TSTP02500	130	191	2
Whatman,Cyclopore, 5 µm pore, 25 mm dia., Qty. 100	Whatman	7062-2513	67	100	3
Hybridisation					
FITC-labelled probe	Thermo Scientific	-	65	95	2
FITC-labelled probe	Oligos Etc.	-	0.7*	1*	3
Igepal CA-630, 50 mL	Sigma Chemical	I3021	16	23	1,3
Polyadenylic acid (poly A) 10 mg/mL	Sigma Chemical	P-9403	29	42	1,3
Deionized formamide, 100 mL	Sigma-Aldrich	F 9037	46	67	2,3
Nonidet-P40, 100mL	Roche	11754599	74	109	2
Counterstaining and microscopical validation					
DAPI (4’-6-Diamidino-2-phenylindole)	Invitrogen, Karlsruhe, Germany	D1306	97	142	2
Slowfade® Gold antifade reagent	Molecular Probes, Invitrogen Detection Technologies	S36936	82	120	1
Citifluor antifade	Citifluor Ltd., Cambridge, UK	X506	117	172	2
ProLong Gold antifade reagent	Molecular Probes, Invitrogen Detection Technologies	P36930	71	104	3

Table 3. Buffers and solution**s**

Solution/Reagent	**Purpose**	**Contents**
Modified Saline Ethanol Solution	Fixative (after Miller and Scholin 2000)	To make up 300 mL: 220 mL 22 vol. 95% ethanol 50 mL 5 vol. Milli-Q water 30 mL 3 vol. 25X SET buffer
Saline Ethanol Solution	Fixative (after Scholin *et al.* 1996)	To make up 300 mL: 250 mL 25 vol. 95% ethanol 20 mL 2 vol. Milli-Q water 30 mL 3 vol. 25X SET buffer
25X SET buffer	Fixative (discard after 12 months)	To make up: 219.15 g 3.75 M NaCl 157.60 g 0.5 M Tris/HCl , pH has to be near 8.0, before adding EDTA 9.3 g 25 mM EDTA, pH 7.8, filter sterilised, 1 Litre
5X SET buffer	Hybridisation buffer (used for diatoms from the genus *Pseudo-nitzschia*)	To make up 400 mL: 316 mL Milli-Q Water 80 mL 25X SET buffer 4 mL 10% Igepal, CA-630 (Sigma Chemical, I 3021)
1X SET buffer	Hybridisation buffer (used for dinoflagellates from the genera *Karenia* and *Alexandrium*)	To make up 400 mL: 380 mL Milli-Q Water 16 mL 25X SET buffer 4 mL 10% Igepal, CA-630 (Sigma Chemical, I 3021)
1X SET buffer, other use	Wash buffer	1X SET buffer in sterile Milli-Q water
0.2X SET	Wash buffer	To process 14 samples: 120 µL 25X SET buffer 14.880 mL Milli-Q water

10 Electrochemical detection of toxic algae with a biosensor

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Introduction

DNA-biosensors (devices that use molecular probes to detect target nucleic acids in a sample) are utilised in a number of different scientific fields. Glucose detection was one of the first applications developed using biosensors (Clark 1956). Electrochemical biosensors combine a biochemical recognition with signal transduction for the detection of specific molecules. The detection component such as a probe sequence, an antibody, an enzyme or other biomolecule, catalyzes a reaction with, or specifically binds to, the target of interest. A transducer component then transforms this detection event into a measurable signal. Different types of biosensors can use optical, bioluminescent, thermal, mass and electrochemical recognition (Gau *et al.* 2005). Currently, biosensors are used in many different applications, such as the identification of infectious organisms, hazardous chemicals and the monitoring of metabolites in environmental samples (Hartley & Baeumner 2003). Biosensors can be produced very cheaply for mass production. A new detection method for the identification of harmful algae is currently being developed using a hand held device and biosensors (Fig. 1 and 2). The first prototype was used to identify the toxic dinoflagellate *Alexandrium ostenfeldii* (Metfies *et al.* 2005). The second prototype, manufactured by Palm Instruments BV (Houten, Netherlands) (Fig. 1), has been extensively used to improve the biosensors. The hand held device "PalmSens" is a compact portable potentiostat for all kinds of electrochemical sensors and cells. It was designed for applications in the field as well as laboratories. It can be configured for a single application but can also be used as a generic electrochemical instrument.

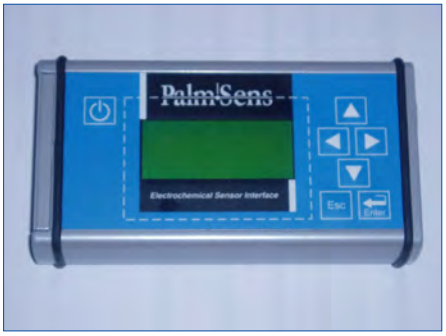

Figure 1. PalmSens device from Palm Instruments.

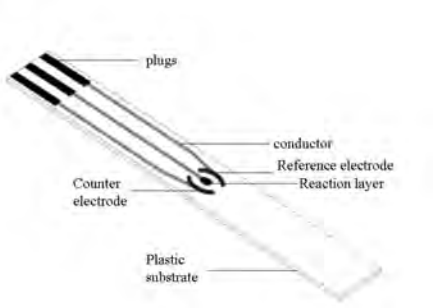

Figure 2. Sensorchip of original prototype with counter and reference electrode and the reaction layer.

Basic principles of electrochemical detection of toxic algae with a biosensor

Molecular probes

Identification of toxic algae is based on oligonucleotide probes that specifically target ribosomal RNA. Targets for the probes are the small and large subunit rRNA genes in the ribosomes of the cells. The conserved and variable regions in these genes make it possible to develop probes specific for different taxonomic levels (Groben *et al.* 2004). The ARB (latin, "arbor" = tree) software package can be used for probe development (Ludwig *et al.* 2004). Theoretical probe specificity is dependent on the number of sequences of the targeted gene available in the databases. If molecular probes are designed from only a few sequences, there is a danger of cross hybridisation to non target species and organisms whose sequences are not in the GENBANK database. Prior to the analysis of field samples, molecular probes have to be tested for specificity with cultivated target species as well as closely related species as *in silico* (calculated by means of a computer simulation) and *in situ* results can show different specificity signals. Nucleic acid probes have been developed for toxic micro-algal taxa includ-

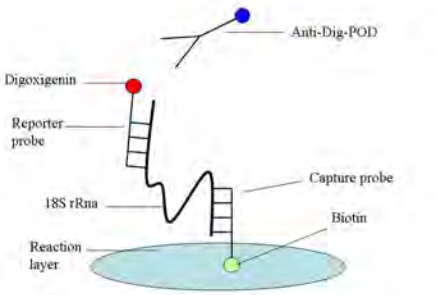

Figure 3. Principle of Sandwich Hybridisation with capture and signal probe.

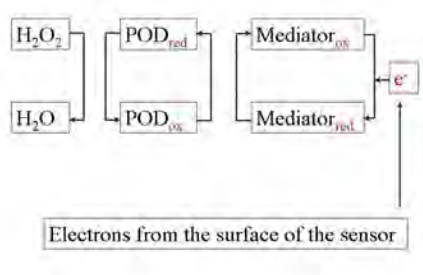

Figure 4. Principle of redox-reaction presenting the electron transport from the surface of the sensor.

The fundamentals of

Electrochemical detection of toxic algae with a biosensor

Scope
The hand held device can be used for the rapid detection of phytoplankton in water samples. The device is a prototype and has been further automated in an EU-funded project (Project ALGADEC).

Detection range
The detection limit has to be determined for each probe set. The calculated detection limit with the hand held device for *Alexandrium ostenfeldii* is ~ 800 cells.

Advantages
Several probe sets have already been developed for this method.

Drawbacks
At the time of publication of this Manual, the hand held device is still a prototype and not available for general purchase. Probes for only a limited number of phytoplankton exist. Probes must be validated for each region where they are applied. Generation of calibration curves are required for each probe set. High sample volume is required if the cell densities are expected to be relatively low. Manual RNA isolation should be done by a trained molecular scientist. The isolation of a sufficient amount of target rRNA from the sample to be tested is required for this assay. A validation of probe signals against total rRNA and over the growth cycle of the algae under different environmental conditions has to be carried out before the method can be applied to field samples.

Type of training needed
Instruction in setting up this technique should come from a person with an in-depth knowledge and experience of molecular biology.
a) perform task: approximately five days are necessary to train an individual in this method.
b) troubleshoot and quality control: a skilled molecular biologist should be available to solve any problems that may arise using this method.

Essential Equipment
Incubator
Thermoheater
Vacuumpump
Washbottle
Mini-Centrifuge
Beadbeater
RNeasy Mini Plant Kit (QIAGEN)
Nanodrop Spectrophotometer
Handheld Device

Equipment cost*
€27350, $38400, for details see Table 4

Consumables, cost per sample**
0.24 € , $0.35 not including probes

Processing time per sample before analysis
Concentration of cells by centrifugation ~20 minutes, coating of electrodes ~ 2 hours and RNA isolation ~1 hour.

Analysis time per sample
Analysis time per sample: Sandwich hybridisation ~ 2 hours.

Sample throughput per person per day
12 to 16 samples per day

No. of samples processed in parallel
Six to eight

Health and Safety issues
Relevant health and safety procedures must be followed. Read Material Data Safety Sheets for all chemicals.
The following three chemicals are particularly hazardous
- Hydrogen peroxide 30 % (H_2O_2)
- N-Phenyl-1,4-benzenediamine hydrochloride (ADPA)
- β-Mercaptoethanol

*service contracts not included
**salaries not included

ing diatoms and dinoflagellates such as *Pseudo-nitzschia* and *Alexandrium* (Scholin *et al.* 1996, Simon *et al.* 1997, Miller & Scholin 1998, John *et al.* 2003).

Disposable sensor-chip and detection principle

The disposable biosensor chip is composed of a carrier material on which a working electrode is printed and the detection reaction takes place, a reference electrode and an auxiliary electrode (Fig. 2).

The working electrode has a diameter of 1 mm and is made of a carbon paste. A biotinylated probe is immobilised on the reaction layer of the working electrode via avidin. The nucleic acids are detected on the sensor chip via a sandwich-hybridisation (Zammatteo *et al.* 1995, Rautio *et al.* 2003) (Fig. 3). The underlying principle of the sandwich hybridisation is that the target specific probe (capture probe) is immobilised via avidin on the surface of the working electrode. If a target nucleic acid is bound to the immobilised probe on the working electrode, the detection of the nucleic acid takes place via a hybridisation reaction to a second target specific probe, the signal probe that is coupled to digoxigenin (Metfies *et al.* 2005).

The digoxigenin specific antibody coupled to horseradish-peroxidase is added to the sensor chip. Horseradish-peroxidase catalyses the reduction of hydrogen peroxide to water. Reduced peroxidase is regenerated by p-aminodiphenylamine (ADPA), which functions as a mediator. The oxidised mediator is reduced at the working electrode with a potential of -150 mV (versus Ag/AgCl) taking an electron from the surface of the sensor (Fig. 4). A potential is applied between the working and the reference electrode. The hand held device measures the resulting current activated through the flow of the electrons from the surface. An electrochemical signal can only be measured if the target nucleic acid binds to both the capture and signal probes (Metfies *et al.* 2005). For each target species the RNA concentration per cell has to be determined. A calibration curve must be developed for each new probe set in order to determine the signal intensity at different RNA concentrations. Using the information on the curve, the electrical measurement of the hand held device can be related to cell numbers in a field sample.

Materials

Laboratory facilities

Fume hood for RNA isolation

Equipment, Chemicals and Consumables

Information on the equipment, chemicals and consumables used in this method are presented in the Appendix, Tables 1-2, at the end of this chapter (Tables 1-2). Suppliers, Catalogue numbers and estimated cost in Euros and US Dollars are also listed in Tables 1-2.

Required Equipment (essential)

- Centrifuge
- Filter, 0.5 µm, ISOPORE™, membrane filters, Millipore, Ireland
- Frit, flask and funnel, Millipore, Ireland
- Mini-Beadbeater™, Biospec products, Biospec products Inc, USA
- Mini-Centrifuge
- Thermoshaker
- Incubator
- Vacuum pump with wash bottle
- Biosensors, Gwent Electronic Materials, Pontypool, UK
- Freezer -80 ºC

Solutions for preservation of microalgal cells

RNAlater, Ambion, Huntingdon, UK

Method

Concentration of cells

Harvesting of cells can be performed by either centrifugation and the supernatant discarded or filtration using a filtration device and a hand held vacuum pump (Fig. 5). A maximum of ~ 1 x 10^7 cells can be processed with the RNeasy Plant Mini Kit.

Preservation and storage

After collecting water samples, the algae cells can be stored at room temperature for several days using RNALater from Ambion, Huntingdon, UK for a later RNA isolation. Follow the instructions that come with RNALater carefully. The cells can also be frozen for long term storage by flash-freezing in liquid nitrogen and immediately transferred to -70 ºC.

RNA Isolation with the RNeasy Plant Mini Kit (QIAGEN) (modified protocol)

General handling of RNA: Ribonucleases (RNases) are very stable, active enzymes and are difficult to inactivate; even minute amounts are sufficient to destroy RNA. All glassware must first be cleaned with a detergent, thoroughly rinsed, and oven baked at 240 ºC for four or more hours before use to avoid any RNase contamination. Gloves must always be worn while handling reagents and RNA samples to prevent contamination from the surface of the skin or from dusty laboratory equipment. Isolated RNA should be stored on ice while being processed.

RNA-Isolation

1 Add 450 µL buffer RLT with ß-ME (ß-Mercaptoethanol) to the cells;
2 Pipette the lysate onto the glass beads and disrupt the lysate in a bead beater for 2x 20 seconds;
3 Pipette the lysate directly onto a QIAshredder spin column (lilac) placed in a 2 mL collection tube, and centrifuge for 15 minutes at maximum speed. Carefully transfer the supernatant of the flow-through fraction to a new microcentrifuge tube without disturbing the cell debris pellet in the collection tube. Use only this supernatant in subsequent steps;
4 Add 0.5 volume (usually 225 µL) ethanol (96–100 %) to the cleared lysate and mix immediately by pipetting. Do not centrifuge. Continue without delay;
5 Apply sample (usually 650 µL), including any precipitate that may have formed, into an RNeasy mini column (pink) placed in a 2 mL collection tube. Close the tube gently and centrifuge for 15 seconds at 8000 x g. Discard the flow-through. Reuse the collection tube in the next step;

Figure 5. Filtration equipment and hand pump.

6. Add 700 µL buffer RW1 to the RNeasy column. Close the tube gently and wait for ca. 45 seconds, then centrifuge for 15 seconds at ≥8000 x g to wash the column. Discard the flow-through and collection tube;
7 Repeat step 6;
8 Transfer the RNeasy column into a new 2 mL collection tube (supplied). Pipette 500 µL buffer RPE onto the RNeasy column. Close the tube gently, and centrifuge for 15 seconds at 8000 x g to wash the column. Discard the flow-through. Reuse the collection tube in step 9.
9 Repeat step 8;
10 Add another 500 µL buffer RPE to the RNeasy column. Close the tube gently and centrifuge for 2 minutes at 8000 x g to dry the RNeasy silica-gel membrane;
11 To elute DNA, transfer the RNeasy column to a new 1.5 mL collection tube. Pipette 30-50 µL RNase-free water directly onto the RNeasy silica-gel membrane. Close the tube gently and centrifuge for 1 minute at 8000 x g to elute;
12 To obtain a higher total RNA concentration, a second elution step may be performed by using the first eluate (from step 11);
13 Measure the RNA concentration by using a spectrophotometer e.g. Nanodrop spectrophotometer.

The isolated RNA should be flash-frozen in liquid nitrogen and immediately transferred to -70 ºC.

Sandwich Hybridisation

A. Coating of Sensor chips

1 The sensor chips are moistened with 50 µL of carbonate buffer (pH 9.6) (Table 1, Fig. 6), which is aspirated off with a vacuum pump (Figs. 7-8);
2 Incubate over night in a moisture chamber at 4 ºC with 2 µL NeutrAvidin in carbonate buffer (500 µg mL^{-1}) (Table 3). Store the electrodes during this period in Petri dishes with moist Whatman filters to protect the solutions from evaporation (Fig. 9);
3 Remove excess NeutrAvidin by washing the chips in PBS (pH 7.6) (Fig. 10, Table 1). Then dry the chips using a vacuum pump attached to a wash bottle;
4 Block the sensors for one hour at room temperature with 20 µL 3 % casein in PBS. Remove the casein by washing with PBS;

Table 1. Buffers for sandwich hybridisation on carbon electrodes.

Buffer	Compound	Concentration
Carbonate buffer (pH 9.6)	$NaHCO_3$	50 mM
10x PBS (pH 7.6)	$NaH_2PO_4 * H_2O$	0.5 M
	NaCl (pH 7.6)	1.54 M
“bead buffer”	NaCl	0.3 M
	Tris (pH 7.6)	0.1 M
4x hybridisation buffer	NaCl	0.3 M
	Tris (pH 8.0)	80 mM
	SDS	0.04%
10x POP buffer (pH 6.45)	$NaH_2PO_4 * H_2O$	0.5 M
	NaCl (pH 6.45)	1 M
PBS-BT (pH 7.6)	PBS	1x
	BSA	0.1 % [w/v]
	TWEEN 20 (pH 7.6)	0.05 % [v/v]

5 The NeutrAvidin coated electrodes can be stored in a fridge for at least 1 year after incubation with 2 % Trehalose in PBS (pH 7.6). The electrodes are coated with 15 µL of Trehalose solution and dried at 37 ºC in an incubator. Before use the electrodes should be washed with PBS (pH 7.6) to remove the Trehalose;

B. Immobilisation of biotinylated DNA-probe

6 Coat the sensor chips with 2 µL of the biotinylated probe [10 pmol $µL^{-1}$ in bead buffer (0.3 M NaCl, 0.1 M Tris)] (Table 1) and incubate for 30 minutes at room temperature;
7 Add 50 µL of 1X hybridisation buffer (0.3 M NaCl, 80 mM Tris, 0.04 % SDS) onto the sensors and directly aspirate off to remove excess unbound probe;
8 The coated electrodes can be stored in a fridge for at least 1 year after incubation with 2 % Trehalose on PBS (pH 7.6). The electrodes are coated with 15 µL of Trehalose solution and dried at 37 ºC in an incubator. Prior to use the electrodes should be washed with PBS (pH 7.6) to remove the Trehalose;

C. Sandwich Hybridisation of immobilized DNA probe, RNA and dioxigenin labelled DNA probe

9 Fragment the RNA by using a fragmentation buffer (200 mM Tris-Acetate, pH 8.1, 500 mM KOAc, 150 mM MgOA). Add 10 µL of rRNA to 2.5 µL fragmentation buffer, heat for 5 minutes at 94 ºC in a thermoshaker (Fig. 11) and immediately chill on ice;
10 Hybridisation preparation details are presented in Table 2. The positive control ensures that the probes are working and the negative control shows the detection of the used compounds that do not contain any target RNA;
11 Heat the preparation for 4 minutes at 94 ºC in a thermoshaker to denature the RNA target strands. Immediately chill on ice:
12 Apply 2 µL of the hybridisation solution onto each sensor. Note that each sample is applied in triplicate;
13 Incubate the chips for 30 minutes at 46 ºC in an incubator then cool the incubator down to room temperature for 5 minutes;

Figure 6: Applying of buffer onto the electrodes.

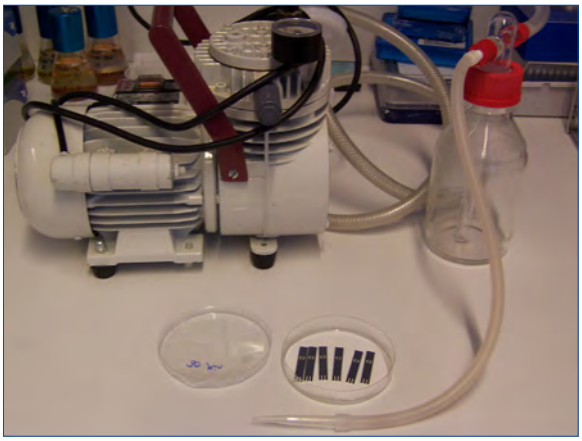

Figure 7. Pump and washbottle for exhausting of buffers from the electrodes.

Figure 8. Drying of chips using a vacuum pump and a wash bottle.

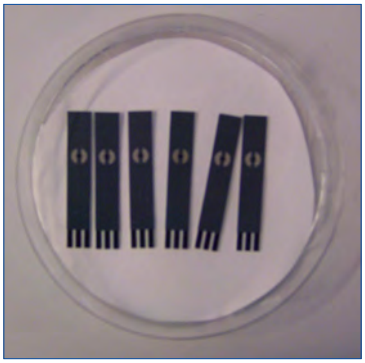

Figure 9. Petri dish with Whatman filter and electrodes.

D. Detection

14 Wash the sensors in 1X POP buffer (pH 6.45) (Table 1) to remove excess RNA;

15 Incubate the sensors with 1.5 µL Anti-Dig-POD [7.5 U mL^{-1} in PBS-BT] at room temperature for 30 minutes (Table 1);

16 Wash the sensors separately in 1x POP buffer to remove excess Anti-Dig-POD and dry with a vacuum pump.

17 Add 20 µL of POD substrate onto the electrode (POD substrate contains 1.1 mg N-Phenyl 1,4-phenylenediamine hydrochloride (ADPA) dissolved in 110 µL ethanol, 250 µL of 100 mM H_2O_2 are added and filled up to 25 mL with 1X POP buffer);

18 Plug the chip into the hand held device and record the measurement (Fig. 12). A summary of the buffers used during the sandwich hybridisation process are presented in Table 1.

Formulas for calculating results

A calibration has to be determined for each probe set to find the signal intensity (nA) for 1 ng RNA. For each target species, the RNA concentration per cell has to be investigated. Subsequently the cell concentration of the target species in a water sample can be calculated from the electrochemical signals:

$$\text{nA (probe signal)} = \text{total ng RNA (present in the sample)}$$

Let then

$$\textit{Cell number} = \frac{\text{nA (probe signal)}}{\text{ngRNA (per cell)}}$$

Quality control

When developing this method it is useful to confirm the result signals and calculated cell counts with real cell count results. This QC system will also determine if the user training provided is sufficient. A molecular biologist should be able to solve any problems that arise during the experiments. False negative signals can result for a number of reasons such as degradation of antibodies or buffers.

Discussion

The electrochemical detection method with the hand held device and biosensors is a rapid method to detect target algae in a water sample. Electrodes can be mass produced. Protocols and electrochemical readings of the hand held device are simple and easy to use, read and interpret. This is useful for people with limited experience with the method.

The hand held device has a standard operating procedure (SOP) with many manual steps. This has now been refined with many improvements resulting from the EU-project ALGADEC. Today, most of the steps are automated (an automated flow and heating chamber for the biosensors) which now allows the detection of 14 species in parallel. The initial sampling, filtering and RNA extraction steps remain the same. The present biosensor consists of a disposable sensor chip with 16 electrodes (Diercks *et al.* 2008a). The redox reaction takes place between the substrate probe and the signal probe to yield a flow of electrons. This allows for a electrochemi-

cal detection proportional to the RNA of the target captured on the chips and thus to the number of cells in the water sample tested. Probes for other toxic algae (e.g., *Alexandrium minutum* and *Gymnodinium catenatum*) have been developed for their detection with this hand held device (Diercks *et al.* 2008b). About 17 different toxic algae can now be detected, along with the negative and positive controls. These newly developed probes are regularly reviewed for their specificity, since new sequences are added to the available online genetic databases daily. This cross check will help to determine if there is any cross reactivity with other marine organisms.

The current detection limit of the hand held device can operate with sample volumes of up to 8-10 Litres which is advantageous if only low cell densities of the target organism are present in the sample. In order to isolate target RNA, an appropriate cell density is required. The detection limit of the hand held device for *Alexandrium ostenfeldii* is ~ 16.00 ng μL^{-1}, with an average yield of ~ 0.02 ng $cell^{-1}$. This equates to ca. 800 cells. A sampling volume of 6.4 l is required to obtain a detectable amount of RNA when the target organism is present at a cell density of 250 cells L^{-1} (Metfies *et al.* 2005).

The manual isolation of RNA is currently the limiting factor of the system. This is because a certain quantity of high quality RNA is required for the assay. It has been found that separate users can isolate different qualities of rRNA from the same sample with an equal number of algae cells present. The resulting signal intensities cannot be compared to cell counts determined using another enumeration technique. Generation of calibration curves is required for each probe set. A validation of probe signals against total rRNA over the growth cycle of the target microalgae under different environmental conditions has to be conducted to verify the calibration curves. This will allow the extrapolation of the electrochemical readings into more accurate values of cells *per* Litre. A high sample volume is required if the cell densities are low. The automated RNA isolation developed under the ALGADEC project will overcome some of these difficulties.

Acknowledgements

Sonja Diercks was supported by the EU-project ALGADEC (COOP-CT-2004-508435-ALGADEC) of the 6th Framework Program of the European Union and the Alfred Wegener Institute for Polar and Marine Research.

Figure 10. Washing of chips in a petri dish with wash buffer.

Figure 11. Schutron Thermoshaker for the fragmentation and denaturation of the sample.

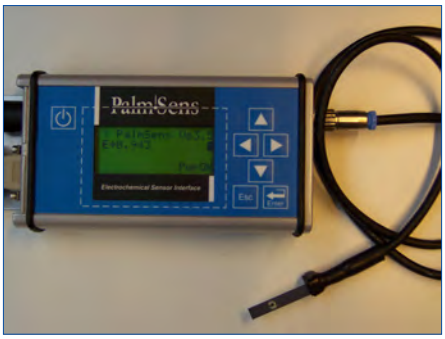

Figure 12. Measuring of chips with Handheld device.

Table 2. Hybridisation preparation.

Detection of the species	Negative control	Positive control
3.5 µL 4X Hybridasation buffer	3.5 µL 4X Hybridasation buffer	3.5 µL 4X Hybridasation buffer
7.5 µL rRNA	1 µL Herring DNA (3480 ng μL^{-1})	1 µL Herring DNA (3480 ng μL^{-1})
1 µL Herring DNA (3480 ng μL^{-1})	1 µL DIG marked DNA probe (1.4 pM μL^{-1})	1 µL Test DNA (36 bases, 1.4 pM μL^{-1})
1 µL DIG marked DNA probe (1.4 pM μL^{-1})	8.5 µL milliQ water	1 µL DIG marked DNA probe (1.4 pM μL^{-1})
1 µl milliQwater		7.5 µl milliQwater

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Appendix

Table 1. Equipment and suppliers.

Equipment	Supplier	Cat. Number	€	US $
Incubator ”Shake’n’Stack”	VWR	7996	2310	3189
Thermoheater ”Comfort”	Eppendorf	5355R	2545	3512
Vacuumpump	Omnilab	9.880540	391	539
Washbottle (250 mL)	Omnilab	5051436	20	25
Mini-Centrifuge Hereaus ”Biofuge pico”	Omnilab	7025235	938	1294
Mini-Beadbeater™	Biospec products, Biospec products Inc, USA	3110BX	715	986
Nanodrop ND1000 Spectrophotometer	Peqlap Biotechnology	91-ND-1000	8995	12418
Handheld Device	Palm Instruments BV, Houten, Netherlands	PalmSens with PC software, Without Pocket PC	3450	4739
Sum approx.			**19364**	**26702**

Table 2. Chemicals and suppliers.

Chemical	**Supplier**	**Cat. Number**	**€**	**US $**
NeutrAvidin™, biotin binding protein	PIERCE, Perbio, Germany	31000	90.00	125.00
D(+)-Trehalose, ≥ 99.5 % HPLC	Fluka BioChemika, Switzerland	90208	31.00	42.64
Biotin-labelled probe (18 bases)	Thermo Electron			
Digoxigenin-labelled probe (18 bases)	Thermo Electron			
Herring-Sperm DNA	Roche Applied Science	10223646001	101.00	138.94
1x PBS	PIERCE, Perbio, Germany	28372	83.65	115.00
Tween 20	Sigma-Aldrich Chemie GmbH, Germany	P1379	26.30	36.18
N-Phenyl 1,4-phenylenediamine hydrochloride $C_{12}H_{12}N_2$ HCl (ADPA, N-Phenyl-1,4-benzenediamine hydrochloride, 1,1-Diphenylhydrazin-hydrochlorid) ADPA	MERCK KGaA, Germany	814648	13.10	18.00
Anti-Digoxigenin-POD fab fragments	Roche Applied Science	11207733910	171.50	235.92
Hydrogen peroxide solution H_2O_2, 30% (w/w)	Sigma-Aldrich Chemie GmbH, Germany	H1009	19.40	26.69
Ethanol	MERCK KGaA, Germany	100983	72.50	99.73
Sodium hydrogencarbonat $NaHCO_3$	Riedel-de Haën®, RdH, Laborchemikalien, GmbH & CoKG, Germany		15.80	21.74
NaH_2PO_4 * H_2O	MERCK KGaA, Germany	567545	29.75	40.92
NaCl	Sigma-Aldrich Chemie GmbH, Germany	S9888	24.80	34.11
Casein	Sigma-Aldrich Chemie GmbH, Germany	C5890	38.00	52.28
Tris (pH 8.0)	Sigma-Aldrich Chemie GmbH, Germany	T1503	101.50	139.63
SDS	Sigma-Aldrich Chemie GmbH, Germany	L4390	18.40	25.40
BSA	Sigma-Aldrich Chemie GmbH, Germany	128910	11.40	15.74
-Mercaptoethanol	MERCK KGaA, Germany	444203	60.00	82.83
RNeasy Plant Mini Kit	Qiagen, Hilden Germany	74904	268.00	370.00
Whatman Chromatography Paper	Whatman, Brentford, United Kingdom	3MM CHR	199.00	274.35
Glass beads (212 – 300 µm, 425 – 600 µm)	Sigma-Aldrich Chemie GmbH, Germany	G1277	99.00	136.19

11 Hybridisation and microarray fluorescent detection of phytoplankton

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Introduction

The introduction of DNA microarray technology in 1995 was one of the latest and most powerful innovations in the field of microbiology. This technique allows the rapid acquisition of copious data (Schena *et al.* 1995). It is a new experimental approach in molecular biology (Blohm and Guiseppi-Elie 2001), which offers the possibility to analyse a large number of samples using a range of different probes in parallel under a diverse spectrum of applications (Ye *et al.* 2001).

Microarray technology was launched with a publication by Schena *et al.* (1995). Many functional genomic methods profit from microarrays, such as genome expression profiling, single nucleotide polymorphism detection and DNA resequencing (Lipshutz *et al.* 1999, Kauppinen *et al.* 2003, Ji and Tan 2004, Yap *et al.* 2004, Al-Shahrour *et al.* 2005, Broet *et al.* 2006, Gamberoni *et al.* 2006). Thus, DNA microarrays are a powerful and innovative tool that can facilitate monitoring in the marine environment.

A microarray consists of DNA sequences that are applied to the surface of a glass slide with special surface properties in an ordered array. It is based on a minimised form of a dot-blot (Gentry *et al*. 2006, Ye *et al*. 2001). A DNA microarray experiment involves microarray production, sample isolation and preparation, hybridisation and data analysis. Prior to the hybridisation, the target nucleic acid is labelled with a fluorescent dye, which can be incorporated directly to the nucleic acid or via indirect labelling of other substances (Cheung *et al.* 1999, Southern *et al.* 1999, Metfies *et al.* 2006). The hybridization pattern is captured via fluorescent excitation in a special device, the microarray scanner (Ye *et al.* 2001).

The application of DNA microarrays for the identification of marine organisms is a relatively new and innovative field of research. It provides the possibility to analyse a large number of targets (species or other taxa) in one experiment (Ye *et al.* 2001), but is not yet widely applied to marine biodiversity and ecosystem science. For the use of microarray technology as a standard tool with rapid and simple routine handling, further research into methodical optimisations is required (Peplies *et al.* 2003).

A number of European research groups utilise DNA microarrays for the identification of marine organisms. The DNA microarrays, or the so called "phylochip", have been used to identify phytoplankton (Metfies and Medlin 2004, Ki and Han 2006, Medlin *et al.* 2006, Gescher *et al.* 2007), bacteria (Loy *et al.* 2002, Peplies *et al.* 2003, Peplies *et al.* 2004a, Peplies *et al.* 2004b, Lehner *et al.* 2005, Loy *et al.* 2005, Peplies *et al.* 2006) and fish (Kappel *et al.* 2003). Specific probes initially developed for other hybridisation assays (e.g. whole cell) have been successfully modified and employed by the microarray detection method.

Basic principles of hybridisation, microarray fluorescent detection

Hybridisation refers to the reannealing of two single strands of nucleic acids that contain complementary sequences. It utilises the basic physical structural property of DNA. DNA has the structure of a double helix with hydrogen bonds binding the two strands of DNA together. When the DNA is heated at a temperature above 90°C, the hydrogen bonds break and the DNA is subdivided in two separate complementary strands. When the temperature is decreased, the two separate strains will reanneal. Complementary nucleic acids with a high degree of similarity anneal easier and will bind together more firmly. The probes on the DNA microarray represent one strand of the DNA and if there are complementary sequences in the examined sample, both stands will hybridise together. This event can be detected by a fluorescence label on one of the two strands that have bound together.

The microarray experiment can be accomplished with ribosomal RNA (rRNA) or DNA, e.g. DNA-fragments generated by a Polymerase Chain Reaction (PCR) from genomic DNA. When using this technique to identify microorganisms rRNA is advantageous over DNA because the cell contains rRNA in a high number that can be easily extracted using commercial kits. In contrast, if the number of copies of ribosomal genes in the genomic DNA is too low then a PCR is needed to amplify the target sequences.

The utilisation of PCR-fragments can introduce a bias to the analyses and it has been shown frequently that microbial communities may not be reflected correctly (Suzuki and Giovannoni 1996, Wintzingerode *et al.* 1997, Simon *et al.* 2000, Speksnijder *et al.* 2001, Kanagawa 2003, Medlin *et al.* 2006). The hybridisation of RNA theoretically offers the possibility of quantification and delivers a less biased view of true community composition (Peplies *et al.* 2006). Possible disadvantages are low yields of RNA from environmental samples and inhibition of extraction by complex organic molecules (Alm and Stahl 2000, Peplies *et al.* 2006). Furthermore, the RNA content can vary over the cell cycle, especially in prokayotes (Medlin 2003, Countway and Caron 2006).

The method requires the use of a molecular laboratory. A clean fume hood should be available because of ß-mercaptoethanol.

DNA microarrays consist of glass microscope slides with particular surface properties (Metfies and Medlin 2005). The

The fundamentals of

The hybridisation, microarray fluorescent detection method

Scope
Detection and quantification of target phytoplankton species. At the time of publication of this Manual, this method is relatively new and currently under development.

Detection range
The detection range depends strongly on the sensitivity of the chosen probes. Calibration curves are required to correlate cell counts with signal point intensities.

Advantages
Allows identification of target phytoplankton to the species level.

Drawbacks
At the time of publication of this Manual, this microarray is still a prototype and not available for general purchase. Probes for only a limited number of phytoplankton exist. Probes must be validated for each region where they are applied. The preparation of a calibration curve is required for each probe used.

Type of training needed
Instruction in setting up this technique should come from a person with an in-depth knowledge and experience of molecular biology.
a) perform task: approximately five days are necessary to train an individual in this method.
b) troubleshoot and quality control: a skilled molecular biologist should be available to solve any problems that may arise using this method.

Essential equipment
A well equipped molecular biology laboratory, see Appendix, Table 1 for details of instrumentation.

Equipment cost*
Total set-up cost = approx. €55,000 or approx. $75,000 US
See Appendix, 2 Table 1 for details.

Consumables, cost per sample**
38 € (50 US $).

Processing time per sample before analysis
A trained person can process up to 8 samples in 6 hours:
RNA Isolation = 1 hour;
Labelling of RNA= 1 hour;
Hybridisation= 4 hours.

Analysis time per sample
A trained person can analyse up to 8 samples in 1 hour.

Sample throughput per person per day
Four samples (with duplicates of each sample) or eight single samples.

No. of samples processed in parallel
Four samples (including duplicates) or eight single samples

Health and Safety issues
Relevant health and safety procedures must be followed.
The following chemical is particularly hazardous: β-Mercaptoethanol.

*service contracts not included
**salaries not included

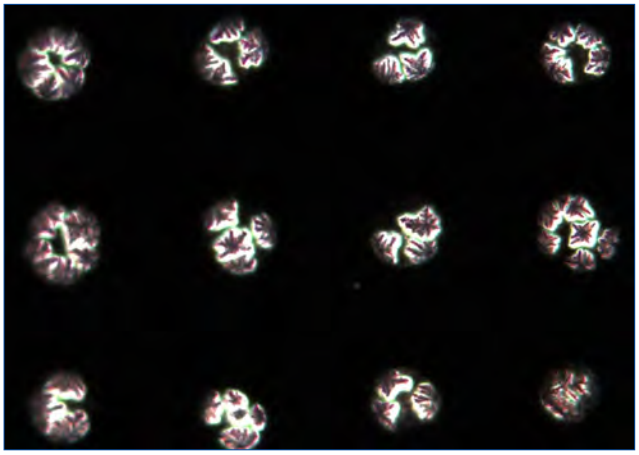

Figure 1. Microscopical view of a part of one glass slide with a grid of 12 spots of probes diluted in 3x SSC salt buffer.

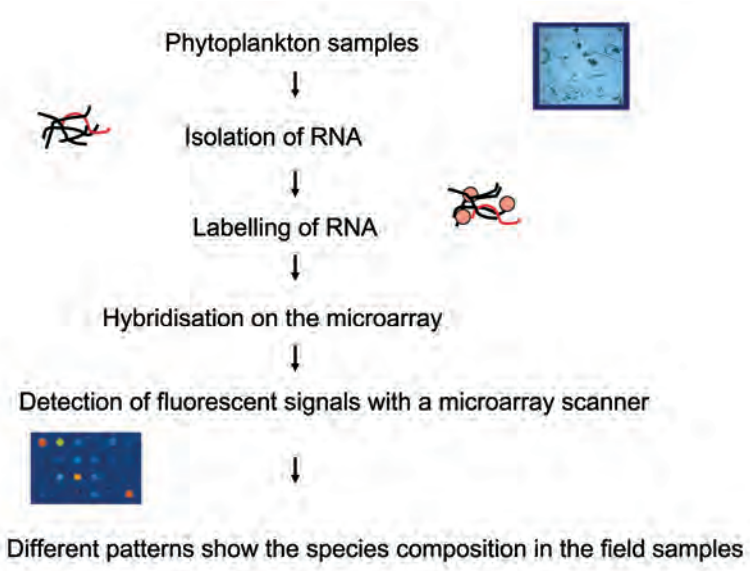

Figure 2. Flowchart of sample handling and hybridisation.

glass slide is coated with a special chemical surface e.g., with aminosilane, an epoxygroup or an aldehydegroup. The probes should be ordered with the appropriate chemical group to be linked to the slide surface. Figure 1 shows a light microscope picture of spots on a glass slide. Furthermore, the probes are immobilised as spots on a glass slide in a defined pattern. Each spot consists of many copies of an oligonucleotide probe that is complementary to a specific target DNA sequence (Graves 1999). The target (RNAs or DNAs) hybridises to the capture oligonucleotide probe on the microarray. The hybridisation is detected via a fluorescent label that is attached to the target sequence during PCR or directly to the RNA (Metfies and Medlin 2004). The flowchart of a microarray hybridisation is shown in Figure 2.

It may be necessary to design new probes or to choose probes from other applications that are specific for the target taxonomic group or species. If the 18S rRNA gene sequence is used to design the probe, then it is important that the probe be designed using only the first 1000 base pairs (bp) of the gene.

Materials

Laboratory Facilities

This method should be carried out in a molecular laboratory with clean fumehood facilities.

Required Equipment (essential)

Microarray production requires the following equipment:

A spotter and an oven that can be heated to 60°C. Ordered probes can be spotted onto a glass slide using a commercial supplier. It is more flexible and convenient to have a spotter in the laboratory. However, these machines are expensive to purchase with prices ranging from €50,000-100,000 (US $67,000-134,000). It is advisable to outsource the spotting procedure at the initial stage of use.

RNA isolation requires the following equipment:

- Mini-Beadbeater (e.g. BioCold Scientific Inc., USA) used to homogenise the algal cells with glass-beads
- Conventional Mini-Centrifuge for small eppendorf tubes (1.0 mL and 1.5 mL)

The hybridisation step requires the following equipment:
- Thermoheater (Fig. 3)
- Incubator
- Bellydancer or shaker (Fig. 4)
- Microarray scanner with software (Fig. 5)

Chemicals and consumables
- RNA isolation, labelling and purification:
- Isolation: RNeasy Plant Mini Kit
- Labelling of RNA: Biotin-ULS-Kit
- Purification of labelled RNA: RNeasy MinElute Cleanup Kit
- Removal of RNAse in fume hood and labware: RNase-Zap (Ambion Inc., Austin, USA)

Information on the equipment, chemicals and consumables used in this method are presented in the Appendix (Tables 1-2) at the end of this chapter. Suppliers, Catalogue numbers and estimated cost in Euros and US Dollars are also listed in Tables 1-2.

Method

Sample Preservation and Storage
1 It is essential to begin with the correct amount of algal material to obtain optimal RNA yield and purity with the RNeasy columns. The required amount depends on the target phytoplankton species and can range from 1 x 10^4 to 1 x 10^7 cells L^{-1};

2 Fresh or frozen tissue can be used. To freeze tissue for long-term storage, the material should be flash-frozen in liquid nitrogen and transfered immediately to -70 ºC where it can be stored for several months. When processing, the tissue should not be allowed to thaw during weighing or handling prior to disruption in Buffer RLT. Homogenised lysates, in Buffer RLT, can also be stored at -70 ºC for several months;

3 To process frozen lysates, thaw samples and incubate for 15-20 minutes at 37 ºC in a water bath to dissolve salts;

4 It is possible to store the hybridised microarrays for at least one year at -20 ºC, although it is usually unnecessary to keep them once they have been scanned. The reuse of "phylochips" for new hybridisations has been tested for expression analysis experiments where the microarrays were reused 5 times (Dolan *et al.* 2001, Bao *et al.* 2002);

RNA Isolation with the RNeasy Plant Mini Kit (Qiagen)
5 Ribonucleases (RNases) are very stable and active enzymes that break down RNA. They generally do not require cofactors to function. As RNases are difficult to inactivate, even minute amounts are sufficient to destroy RNA. Plasticware or glassware, therefore, should not be used without first eliminating any possible trace of RNase contamination. Care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the isolation procedure. Latex or vinyl gloves should always be worn when handling reagents and RNA samples to prevent RNase contamination from the skin surface or dusty laboratory equipment. Gloves should be changed frequently and tubes kept closed whenever possible. Samples should be kept on ice, particularly isolated RNA, especially when aliquots are being pipetted. The use of sterile, disposable polypropylene tubes is recommended throughout. These tubes are generally RNase-free and do not require a pre-treatment to inactivate RNases. Glassware used for RNA work should be cleaned with a detergent, thoroughly rinsed and oven baked at 240 ºC for 4 or more hours before use;

Figure 3. Thermoheater for denaturation of the labelled RNA.

Figure 4. Washing of the microarrays on a belly dancer.


Figure 5. Microarray scanner GenePix 4000B.

Important notes before getting starting
6 Beta-Mercaptoethanol (ß-ME) must be added to Buffer RLT before use. Beta-Mercaptoethanol is toxic so dispense in a fume hood and wear appropriate protective clothing. Add 10 µL ß-ME per 1 mL Buffer RLT. Buffer RLT is stable for 1 month after the addition of ß-ME;

7 Buffer RPE is supplied as a concentrate. Before using for the first time, add 44 mL of ethanol (96-100 %), as indi-

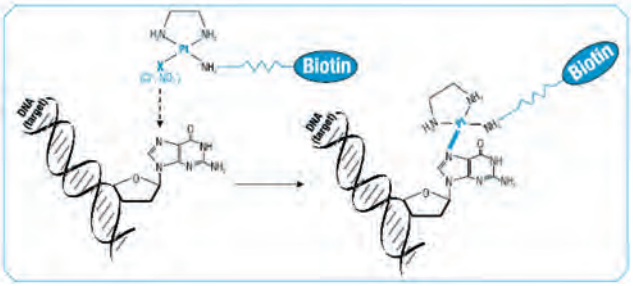

Figure 6. Labelling of nucleic acids with Biotin-ULS (source: www.fermentas.com)

cated on the bottle, to obtain a working solution.

8 All steps of the RNeasy protocol should be performed at room temperature (~20 °C). Work quickly during the procedure;

9 All centrifugation steps are performed at 20-25 °C in a standard microcentrifuge;

Harvesting of phytoplankton cells

10 Harvest the cells by centrifugation or filtration;

11 Discard the supernatant and process the cell pellet;

RNA-Isolation

12 Add 450 µL Buffer RLT with ß -ME to the cell pellet;

13 Pipette the cells into an eppendorf cup containing glass beads (212 µm- 300 µm and 312-600 µm) and homogenise the cells in a bead beater for 20 seconds;

14 Pipette the lysate directly onto a QIAshredder spin column (lilac colour) placed in 2 mL collection tube and centrifuge for 2 minutes at maximum speed;

15 Carefully transfer the supernatant from the flow-through fraction to a new microcentrifuge tube without disturbing the cell debris pellet in the 2 mL collection tube. This supernatant/lysate is used in all subsequent steps;

16 Add 0.5 volume (usually 225 µL) ethanol (96-100 %) to the clear lysate, and mix immediately by pipetting. Do not centrifuge. Continue without delay;

17 Apply the sample (usually 650 µL), including any precipitate that may have formed, to an RNeasy mini column (pink colour) placed in a 2 mL collection tube. Close the tube gently and centrifuge for 15 seconds at ≥8000 x g (≥10000 rpm). Discard the flow-through;

18 Reuse the collection tube in the next step;

19 Add 700 µL Buffer RW1 to the RNeasy column. Close the tube gently and centrifuge for 15 seconds at ≥8000 x g (≥10000 rpm) to wash the column. Discard the flow-through and collection tube;

20 Transfer the RNeasy column into a new 2 mL collection tube (supplied with the kit). Pipette 500 µL Buffer RPE onto the RNeasy column. Close the tube gently and centrifuge for 15 seconds at ≥8000 x g (≥10000 rpm) to wash the column. Discard the flow-through;

21 Add another 500 µL Buffer RPE to the RNeasy column. Close the tube gently, and centrifuge for 2 minutes at ≥8000 x g (≥10000 rpm) to dry the RNeasy silica gel membrane;

22 To elute the RNA, transfer the RNeasy column to a new 1.5 mL collection tube. Pipette 30-50 µL RNase-free water directly onto the RNeasy silica gel membrane. Close the tube gently and centrifuge for 1 minute at ≥8000 x g (≥10000 rpm) to elute;

23 To obtain a higher total RNA concentration, a second elution step may be performed by using the first eluate (from the previous step). Pipette the eluate back on the column and centrifuge for 1 minute at ≥8000 x g (≥10000 rpm) to elute once again;

24 Measure the concentration of the RNA with a Spectrophotometer (e.g. Nanodrop Spectrophotometer, Peqlab, Erlangen, Germany).

Labelling of RNA with the Biotin-ULS-Kit

Background: This Labelling Kit uses the Universal Linkage System (ULS) technique, which is based on the stable coordinative binding of a platinum complex to nucleic acids. The platinum complex acts as a linker between a detectable marker (label) molecule, i.e., fluorescein or biotin, and DNA or RNA. The marker is coupled directly to the nucleic acid without any significant interference. Universal Linkage System consists of a Pt complex stabilised by a chelating diamine. It has two binding sites, one of which is used to bind a marker. The other binding site is used to link the complex to the aromatic nitrogen atoms of nucleobases and one nitrogen atom of guanine is strongly preferred (Fig. 6). The resultant Pt-N bond is very stable both chemically and thermally.

Features of the Biotin-ULS-Kit

- One-step reaction.
- Fast - only 30 minute to label the target.
- Universal - any nucleic acid, independent of size or structure can be labelled.
- Easy to scale up and down. It allows labelling of as little as 25 ng or as much as 10 µg of nucleic acid in a single reaction

25 Add 1 µL (= ½ U) of Biotin ULS reagent to 500 ng of nucleic acid template;

26 Adjust volume with labelling solution to 20 µL and mix well;

27 Incubate for 30 minutes at 85 °C;

28 Add 5 µL Stop solution and mix well;

29 Incubate for 10 minutes at room temperature;

30 Purify the solution with the RNeasy MinElute Cleanup Kit before hybridisation;

Purification of labelled RNA with the RNeasy MinElute Cleanup Kit (Qiagen)

31 Adjust sample to a volume of 100 µL with RNase-free water. Add 350 µL of Buffer RLT and mix thoroughly;

32 Add 250 µL of 96-100 % ethanol to the diluted RNA and mix thoroughly by pipetting. Do not centrifuge. Continue the next step immediately;

33 Apply 700 µL of the sample to an RNeasy MinElute Spin Column in a 2 mL collection tube. Close the tube gently and centrifuge for 15 seconds at ≥8000 x g (≥10000 rpm). Discard the flow-through;

34 Transfer the spin column into a new 2 mL collection tube. Pipette 500 µL Buffer RPE onto the spin column. Close the tube gently and centrifuge for 15 seconds at ≥8000 x g (≥10000 rpm) to wash the column. Discard the flow-through;

35 Add 500 µL of 80 % ethanol to the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 2

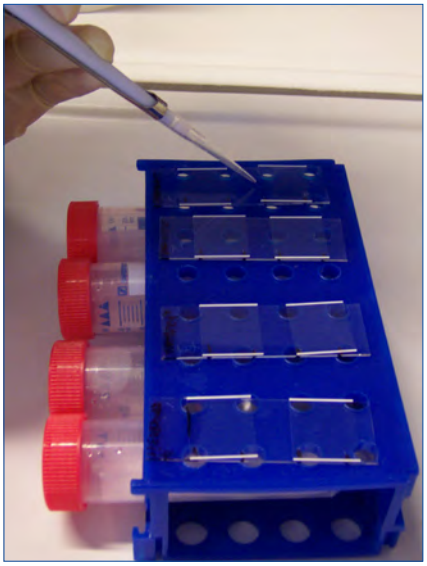

Figure 7. Pipetting of hybridisation solution on one microarray.

minutes at ≥8000 x g (≥10000 rpm) to dry the silica gel membrane. Discard the flow through and collection tube;

36 Transfer the RNeasy MinElute Spin Column into a new 2 mL collection tube. Open the cap of the spin column and centrifuge in a microcentrifuge at full speed for 5 minutes. Discard the flow through and collection tube;

37 To elute the RNA, transfer the spin column to a new 1.5 mL collection tube. Pipette 14 µL RNase-free water directly onto the centre of the silica-gel membrane. Close the tube gently and centrifuge for 1 minute at maximum speed to elute;

38 To obtain a higher total RNA concentration, a second elution step may be performed by using the first eluate (from the previous step);

39 Measure the concentration of the RNA with a Spectrophotometer;

Microarray Hybridisation

40 The positive control in the microarray hybridisation experiment is a probe (ATGGCCGATGAGGAACGT) specific for a 250 bp fragment of the TATA-box binding-protein (TBP) gene of *Saccharomyces cerevisiae* (Metfies and Medlin 2004);

41 The gene is amplified with the primers TBP-F (5'-ATG GCC GAT GAG GAA CGT TTA A-3') and TBP-R-Biotin (5'-TTT TCA GAT CTA ACC TGC ACC C- 3') and is added to the hybridisation solution;

42 A negative control probe that has no match to any sequence found in the NCBI (National Center for Biotechnology Information) database should be used e.g. TCCCCCGGGTATGGCCGC (Metfies and Medlin 2004);

43 Pre-hybridisation step: incubate the microarrays in a microarray box containing ~ 20 mL pre-hybridisation buffer [1X SET / 1 mg mL^{-1} BSA] for 60 minutes at the predetermined hybridisation temperature (58 °C). Subsequently wash the microarrays briefly in deionised water and by centrifugation;

44 Apply 30 µL of the 40 µL hybridisation solution to the microarray. The solution contains labelled target nucleic acid dissolved in hybridisation buffer. The final concentration of the target nucleic acid should be ~ 10 ng μL^{-1}. The solution should also contain the positive control (i.e. 250 bp PCR-fragment TBP from *S. cerevisiae* with biotin-labelled primers) in a final concentration of 4.7 ng μL^{-1};

45 Incubate the hybridisation solution for 5 minute at 94 °C in a thermoheater (Fig. 3) to denature the target nucleic acid. The use of a special kind of coverslip, the Lifter Slip coverslip (Implen, München, Germany) is recommended to ensure an even dispersal of the hybridisation mixture onto the microarray;

46 Place the cover slip on the slide and pipette 30 µL of the hybridisation mixture under the cover slip (Fig. 7);

47 Hybridise at 58 °C for 1 hour in a wet chamber. A 50 mL Falcon-tube with a wet Whatman paper functions very well as a moisture chamber. Apply approximately 1 mL of hybridisation buffer onto the Whatman paper to obtain enough humidity in the chamber;

48 After the hybridisation is complete, remove excessive target nucleic acid and unspecific bindings by stringent washing of the microarrays. Remove the cover slip from the array and put the microarray into a Falcon-tube with 50 mL wash buffer 1. Place the Falcon-tube on a belly dancer and shake for 10 minutes (Fig. 4). Repeat this step with wash buffer 2. Wash-buffer 1 contains: 2X SSC / 10 mM EDTA / 0.05 % SDS; wash-buffer 2: 1X SSC / 10 mM EDTA;

49 Wash again for 5 minutes with increased stringency [wash-buffer 3: 0.2X SSC / 10 mM EDTA]. Whereas the first washing buffer contains SDS, it is recommended that the next 2 washing buffers do not contain SDS. This is because residual SDS will generate high background intensities on the microarray;

50 Remove the last wash buffer and dry the microarrays by centrifugation in the falcon-tube (approx. 3 minutes at approx. 2000 rpm). There are also special microarray centrifuges with only the optimal speed;

Fluorescent Staining of the microarrays

51 Visualise the hybridised biotinylated target nucleic acids by staining the microarray for 30 minutes at room temperature in a wet chamber containing 50 µL Streptavidin-Cy5 [0.1µg mL^{-1} Streptavidin / 1X hybridisation buffer];

52 Remove excess stain by washing the microarrays twice for 5 minutes with wash-buffer 1 and once for 5 minutes with wash-buffer 2. Carry out the washing steps at room temperature and place the microarrays in a 50 mL Falkon-tube;

53 Dry the microarrays by centrifugation (approx. 3 minutes at approx. 2000 rpm);

Analysis

54 Scan the microarray using the GenePix Axon 4000B scanner at 635 nm;

55 Analyse the obtained signal intensities with the GenePix 6.0 software;

56 Calculate the signal to noise-ratios according to Loy *et al.* (2002);

57 All calculated ratios should be normalised to the signal of the TBP positive control. A schematic picture of the excitation is shown in Figure 8.

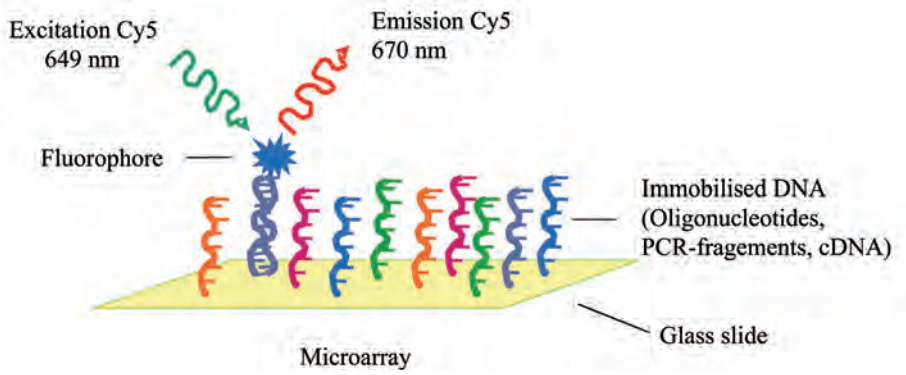

Figure 8. Microarray detection.

Formula for calculating results

$$Cell\ concentration\ (cells\,L^{-1}) = \left(\frac{Positive\ cell\ count\ on\ whole\ filter\ (N)}{Volume\ of\ sample\ (mL)}\right) * 1000$$

Discussion

The utilisation of molecular methods has increased the potential for investigating the biodiversity of phytoplankton in the marine environment. The identification of phytoplankton, especially of harmful algal species, is important from an ecological and economic point of view. Microorganisms dominate global biological diversity, in terms of their species numbers, but their small size and lack of morphological features make it difficult to assess their overall biodiversity.

In the past, regular monitoring of phytoplankton has been hampered by the lack of reliable morphological features in some groups of species. Even with the introduction of electron microscopy, it is difficult to make the correct classification, especially in picoplanktonic taxa or with hidden genetic diversity in morphological indistinguishable species (Scholin 1998, Zingone *et al.* 1999, Massana *et al.* 2002, Janson and Hayes 2006). As a result knowledge of the complexity of the phytoplanktonic ecosystems is still limited. Many species are sensitive to sample fixation (Gieskes and Kraay 1983) and some possess different life stages with varying morphological properties (Partensky *et al.* 1988). The expertise of the scientist may also influence the identification (Bornet *et al.* 2005, Godhe *et al.* 2007).

The utilisation of microarrays for the detection and monitoring of marine microalgae, although a relatively new technique, has already undergone several trials (Metfies and Medlin 2004, Ki and Han 2006, Medlin *et al.* 2006, Gescher *et al.* 2007). Previous work has shown that microarrays can be used for the identification of phytoplankton using 18S rDNA probes at the class level (Metfies and Medlin 2004, Medlin *et al.* 2006). Ki and Han (2006) and Gescher *et al.* (2007) have also demonstrated the specificity of 18S and 28S rDNA probes for the detection of harmful algae at the species level.

One drawback of the method is the dependency on the sequence database for probe design. It is estimated that the known rRNA sequence database is only a very small fraction of the overall biodiversity present in the environment. The number of 18S rRNA sequences in public databases is constantly growing. Developed probes should be regularly checked against all known sequences to ensure cross reactivity with a non target organism does not occur.

The the detection limit of the microarray depends on the sensitivity of the chosen probes. In general, a high sampling volume of up to several Litres of seawater is required. This is especially true if the number of cells present in the sample is low. Another limiting factor of the microarray method lies in the manual isolation of RNA. The manual isolation of RNA from a set number of target cells can vary depending on the skill of the operator. This can result in different signal intensities and so the resulting cell counts cannot be compared. The utilisation of an automatic device (e.g. pipetting robot) for RNA isolation may resolve this problem. The isolation of a sufficient amount of RNA is very important because the target RNA presents only a small fraction of the RNA isolated from a wild sample.

A calibration curve must be developed for each new probe to monitor individual algal species. The signal must be validated with cell numbers. A good correlation of cell counts and RNA concentration per cell with signal intensity is prerequisite for a reliable analysis of field samples. Different physiological conditions may have an influence on the RNA content per cell.

The development and evaluation of microarrays is time-consuming and costly, but once a microarray is well developed, it is a cost-effective, trusted and efficient tool to detect the target organism. Limitations and cost aside, the use of microarrays to answer ecological and biodiversity questions offers for the first time the possibility to analyse samples with a large number of different targets (species or other taxa) in parallel (Ye *et al.* 2001). One of the most likely future uses of microarrays in the field of phytoplankton ecology is to monitor the biodiversity of phytoplankton over long-time scales (Medlin *et al.* 2006, Gescher *et al.* 2007c).

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Appendix

Table 1. Equipment and suppliers.

Equipment	Supplier	Cat. Number	€	US $
Mini-Beadbeater™	Biospec products, Biospec products Inc, USA	3110BX	715	986
Mini-Centrifuge Hereaus ”Biofuge pico”	Omnilab, Germany	7025235	938	1294
Nanodrop ND1000 Spectrophotometer	Peqlap Biotechnology, Germany	91-ND-1000	8995	12418
Incubator ”Shake’n’Stack”	VWR, Germany	7996	2310	3189
Thermoheater ”Comfort”	Eppendorf, Germany	5355R	2545	3512
Shaker (Bellydancer)	Sigma Aldrich, Germany	Z36, 761-3	1400	1800
Scanner and software (GenePix 4000B device and GenePix Pro.6.0 software)	Molecular Devices Corporation, USA	97-0002-00	40000	51000
Sum approx.			**56903**	**74199**

Table 2. Chemicals and suppliers.

Chemical	Supplier	Cat. Number	€	US $
Spotting (per slide)	Commercial supplier or with own spotting device	-	75	100
RNeasy Mini Plant Kit	Qiagen Inc., USA	74904	268	370
Biotin-ULS Kit	Fermentas Inc., USA	K0631	500	640
RNeasy MinElute Cleanup Kit	Qiagen Inc.,USA	742043	235	300
EDTA	Sigma Aldrich, Germany	E5134	80	102
Citric Acid	Merck KGaA, Germany	231211	25	32
Sodium chloride NaCl	Sigma Aldrich, Germany	S9888	23	29
SDS	Merck KGaA, Germany	L4390	40	51
Bovine Serum Albumin BSA	Sigma Aldrich, Germany	A2153	30	38
Trizma Base	Sigma Aldrich, Germany	T_1503	80	102
Triton x-100	Merck KGaA, Germany	648466	33	42
Ethanol, pro Analysis	Merck KGaA, Germany	1,009,832,500	73	93
1 probe (18 bases, with Aminolink) + Positive control, Negative control	Thermo Electron, Gemany	-	100	127
Herring-Sperm DNA	Roche, Germany	223646	91	116
Streptavidin-CY5	KPL, USA	079-30-00	250	319

12 Toxic algal detection using rRNA-targeted probes in a semi-automated sandwich hybridization format

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Introduction

The Sandwich Hybridisation Assay (SHA) provides a simple and rapid means to detect and estimate cell density of a variety of algal species associated with harmful algal blooms (HABs). Results from the SHA system can discriminate to species level using both cultured and natural samples. Having this level of discrimination without the need for microscopy and advanced training in taxonomy, gives researchers, public health officials and water quality managers a powerful tool to rapidly assess changing HAB communities. The SHA system uses species-specific, ribosomal RNA (rRNA) targeted DNA probes that are applied using a semi-automated robotic processor (Scholin *et al.* 1996, 1997, 1999, Greenfield *et al.* 2008). Currently, DNA probes for *Pseudo-nitzschia* spp., *Alexandrium* spp., *Heterosigma akashiwo*, *Chattonella* spp., and *Fibrocapsa japonica* are available (Scholin *et al.* 2004). Others probes include those for *Coccholodinium polykrikoides* (Mikulski *et al.* 2008), a variety of *Karenia* spp., *Karlodinium veneficum* and *Gymnodinium aureolum* (Haywood *et al.* 2007). In New Zealand the SHA method has gained international accreditation and is used to regulate shellfish harvests (e.g. Ayers *et al.* 2005 and references therein). Recent progress made on assays for invertebrates (Goffredi *et al.* 2006), including the invasive European green crab (Jones *et al.* 2008), and marine bacteria (Preston, 2009) offer opportunities for use of the SHA format to detect many other organisms as well.

Basic Principles of Sandwich Hybridisation

The SHA referred to here (after Scholin *et al.* 1996, 1999, Greenfield *et al.* 2008) employs two DNA probes that target ribosomal RNA (rRNA) sequences. Assays for both the large and small subunit (LSU, SSU) rRNA have been implemented. Assays are performed using pre-filled 96 well microplates and a robotic processor supplied by Saigene Biotech Inc. A capture probe complementary to a variable sequence is attached to a mechanical solid support (prong in a sandwich hybridisation machine), which is then submerged into the prepared sample and hybridizes with the target molecule if present. Captured molecules are then washed to remove any unbound material. To detect the captured molecules, a second hybridisation step is initiated using a DNA probe conjugated to a signal probe. This probe is targeted to a more conserved region of the captured fragment. The resulting "sandwich" of capture probe/target molecule/signal probe is detected using an enzymatically-driven colorimetric reaction. Figure 1 provides a schematic view of the sandwich hybridisation chemistry described above (for details see Greenfield *et al.* 2008).

The basic steps of the SHA method are:

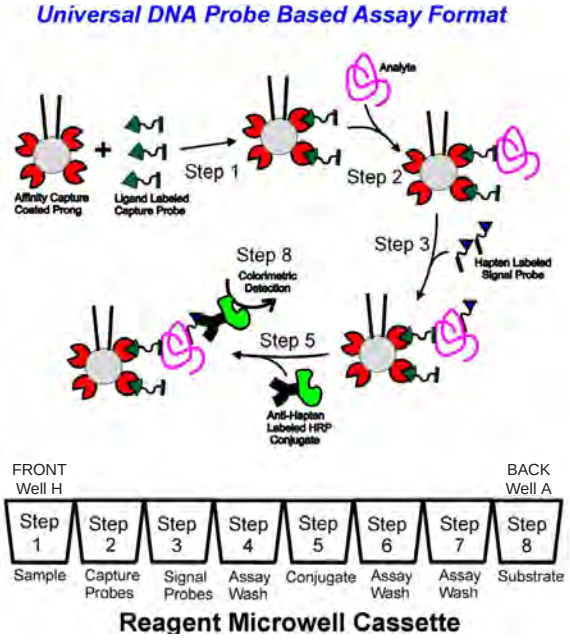

Figure 1. Schematic view of the sandwich hybridisation chemistry

1 Collect sample onto a filter,
2 Lyse sample using a chaotropic buffer (disrupts and denatures the 3-D structure of macromolecules) and heat,
3 Filter lysate,
4 Load sample lysate into 96-well plate,
5 Run SHA processor (automated),
6 Record colour development (O.D. 650 and 450nm),
7 Compare results against standard curves to estimate abundance of target species.

Laboratory Facilities

The SHA system requires a typical laboratory setting that is protected from direct sunlight, excessive dust, and temperature extremes.

Essential Equipment

(for more details see Appendix, Table 1 at the end of this chapter)

- Bench top processor (after Scholin *et al.* 1999)
- 96-well Microplate reader that can read wavelengths 650 nm and 450 nm
- Software to record microplate data and apply data conversion algorithm
- Refrigerated storage (2° to 8°C)
- Vacuum filter manifold
- 85 °C heat block
- 12-channel multiple pipette (30-300 µL)

The fundamentals of

The sandwich hybridisation method

Scope
Detection and quantification of a target phytoplankton species using ribosomal RNA-targeted, DNA probe-based assays.

Detection range
Detection performance is unique to each probe set used in the SHA system.

Advantages
The SHA system provides a robust and simple semi-automated method to detect and estimate cell abundances of target species.

Drawbacks
Probes are only available for a limited number of target species. Specificity of probes must be established on a regional basis. This system may not be suitable for detection of very rare target sequences.

Type of training needed
Instruction in setting up this technique should come from a person with an in-depth knowledge and experience of molecular biology. A minimum of three days are needed for to cover theory, operation, data processing, etc. A skilled molecular biologist should be available to solve any problems that may arise using this method.

Essential Equipment
SHA semi-robotic processor, microplate reader, heating block, filtration manifold, 12-channel and single channel micropipettors.

Equipment cost*
SHA semi-robotic processor, €5139 (US $7500)
Total set-up cost = €14197 (US $20666).
See Appendix, Table 1 for details

Consumables, cost per sample**
€5-7 (US $7-10).

Processing time per sample before analysis
15-20 minutes (hands-on)

Analysis time per sample
75 minutes (hands-off).

Sample throughput per person per day
30-40 samples in an 8 hour day given 1 processor.

No. of samples processed in parallel
6, 8, or 12 samples with replicates 4, 3, or 2, respectively.

Health and Safety issues
Relevant health and safety procedures must be followed. The lysis and signal probe buffers contain guanidine thiocyanate, which can damage skin and eyes.

*service contracts not included
**salaries not included

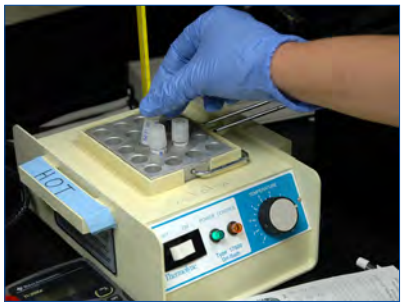

Figure 2. Heating block set to 85ºC used for sample lysis.

Figure 3. Three-position vacuum filter manifold used to collect particulate fraction of water sample.

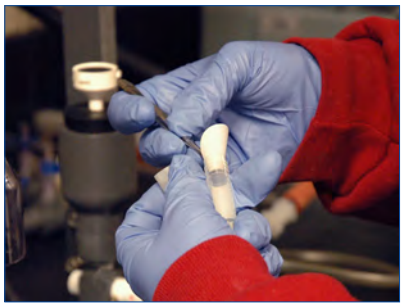

Figure 4. Filter, sample side in, being placed in cryovial for archival and/or lysis.

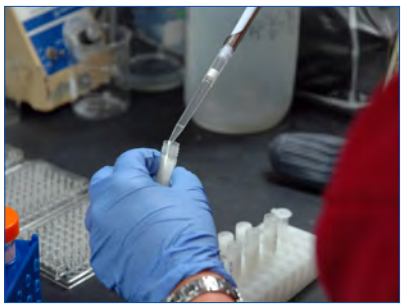

Figure 5. Addition of lysis buffer to cryovial with filter.

Figure 6. After lysis, lysate is syringe filtered into clean tube.

- 100-1000 µL single channel pipette
- Cold storage for samples: liquid nitrogen is preferred, -80ºC and –20ºC (e.g., freezer or dry ice) can also be effective for storing archival samples (filters)
-

Chemicals and Consumables

(for more details see Appendix, Table 2 at the end of this chapter)

- 25mm hydrophilic Durapore filter
- 2mL cryovial
- 13mm syringe filter
- 5cc syringe
- Polypropylene tubes 12X 75mm
- 10% H_2SO_4
- filling trays/pipette tips

Probes

The suite of SHA probes currently available for HAB spp. include the following:

Diatoms

Pseudo-nitzschia australis (and other diatoms below, Scholin *et al.* 1999)
Pseudo-nitzschia multiseries
Pseudo-nitzschia pungens
Pseudo-nitzschia pseudoelicatissima/multiseries complex

Dinoflagellates

Alexandrium tamerense/catenella/fundyense (North American ribotype, Matweyou *et al.* 2004, Anderson *et al.* 2005)
Cochlodinium polykrikoides (Mikulski *et al.* 2008)
Gymnodinium aureolum (and other dinoflagellates below, Haywood *et al.* 2007)
Karenia brevis
Karenia mikimotoi
Karenia selliformis
Karenia papilionacae
Karlodinium veneficum

Raphidophytes

Heterosigma akashiwo (and others below, Tyrrell *et al.* 2001, 2002, Scholin *et al.* 2004)
Fibrocapsa japonica
Chattonella antiqua/subsalsa

Method

Preparation to run samples

1 Turn on heating blocks for sample lysis (Fig. 2) and processor and check that desired temperatures are at their proper values. Lysis is carried out at 85 ºC. The processor plate should provide a temperature of 28-30 ºC.
2 Obtain necessary lysis tubes for runs or alternatively label 2 mL cryovials for storage of sample filters in liquid nitrogen for later analysis (see below).
3 Start the microplate reader.
4 If using pre-made plates, remove seal and let it reach room temperature protected from light and dust. If required, dispense reagents into 96-well microplate as shown in the instruction booklet that comes with materials supplied by Saigene Corporation (see also Greenfield *et al.* 2006). When dispensing reagents to a microplate use barrier tips

to minimise cross-well contamination. Dispense 0.25 mL per well. Do not blow out small amounts of fluids following primary delivery of reagents and samples to the plate or excessive bubbles will form in the well; bubbles can interfere with the assay.

Sample, plate and prong handling

5 Protect plate and sample from sunlight and excessive heat. Samples should be filtered and lysed as soon as possible, or filters can be archived for later analysis by rolling the filter into a cryovial (particles away from the tube wall and freezing the filters in liquid nitrogen or alternative cold storage). Use plate within 1 hour after removing seal and/or dispensing reagents. Prongs should remain in package until used. Handle prongs with forceps touching only the strip, or backbone, that connects the 12 prongs; avoid touching the prongs themselves. Store prongs at 4-8 ºC with packaging and desiccant provided.

Sample filtration

6 Samples should be collected using a vacuum manifold at a vacuum pressure of approximately 100-150 mmHg (Fig. 3). Filter samples onto hydrophilic Durapore filters (generally 0.65-0.45 µm pores size; Millipore). The volume filtered should be no more than what can pass through the filter in about 20 minutes. Typical sample volumes are 200 to 400 mL of whole water. Samples that have been pre-concentrated (e.g. net tow or sieve) can also be collected on the Durapore filter as well.

Archival and Lysis of Sample

7 After filtering, place filter membrane into a 2 mL cryovial. Place the filter with the sample side facing away from the tube wall. Do not crumple the filter and be sure to push the filter to bottom of the tube (Fig 4). If sample is to be archived, cap the 2 mL cryovial and store in liquid nitrogen without lysis buffer.

8 To process, add 1-2 mL of lysis buffer to cryovial with filter and cap it tightly (Fig 5). Place cryovial in heating block with wells half filled with water to enhance heat transfer (Fig. 2). Heat for 5 minutes total with a brief finger vortex after 2.5 minutes.

9 After heating, allow lysate to cool for 5 minutes. Use lysate within 20 minutes of preparation.

10 Remove plunger from a 5 cc disposable syringe (Becton and Dickinson).

11 Install a 13 mm, 0.45 µm Millex-HV (Millipore) filter onto the syringe.

12 Place tip into a clean polypropylene collection tube, add lysate to syringe barrel and push lysate through filter until foam appears at the tip of filter (Fig. 6). Lysates from replicate samples may be combined to yield a larger volume of lysate from a given sample.

13 The lysate is now ready to be loaded onto the plate sample well (row H).

Processing

14 Load 0.25 mL filtered lysate per sample well (Fig. 7).

15 Load prong onto processor arm and secure spring clip. Do not handle prong with bare hands. Hold prong by the backbone with tweezers and avoid scraping the prongs against the processor arm.

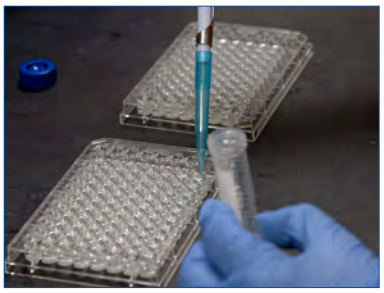

Figure 7. Lysate (0.25 mL) is added to appropriate sample wells on microplate.

Figure 8. Microplate being placed on processor after prongs added to processor arm.

Figure 9. Processor being started after plate is positioned on heater base.

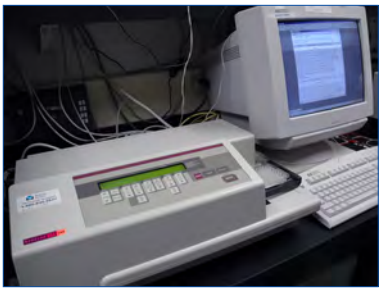

Figure 10. Microplate is placed on microplate reader cradle; optical density is recorded at 650nm for row “A” (wells in which colour development has occurred).

Figure 11. After microplate is read at 650nm, row “A” is acidified with 50 µL of 10% H_2SO_4;optical density is recorded at 450 nm for row “A”. The measure at 650 nm is considered low sensitivity while that at 450 nm is considered high sensitivity. For positive samples, the optical density at 450 nm should be roughly 2X that at 650 nm.

16 Mount 96 well microplate onto processor, make sure to centre the microplate on the heating plate (Fig. 8).
17 Push the "RUN" button (Fig. 9) and make sure that the prongs enter the wells without touching the sides of the wells. The plate will be ready to read in just over an hour. A digital timer will display time remaining; the counter will read "0" approximately 90 seconds before the process is actually complete. The processor will display a message indicating that the reaction is complete, and plate is ready to be read.

Reading the Plate

18 When the processor is finished, immediately read the plate(s) (Fig. 10).
19 First read the plate at 650 nm.
20 Next acidify row A with 50 µL of 10 % H_2SO_4 (add and mix using a 12-channel pipette and avoid introduction of bubbles) (Fig. 11).
21 After all bubbles have popped (~10-45 seconds) read plate at 450 nm.

Data Processing

To calculate the concentration of target in a sample, the assay requires that you establish an empirically derived dose response curve that relates optical density (O.D.) to a known number of cells per well of lysate (see Greenfield *et al.* 2008, Haywood *et al.* 2007, Ayers *et al.* 2005 for details in establishing a dose response curve). Using optical density (from the assay), the dose response curve, sample volume and lysis buffer volume (used to lyse the sample) you can estimate the target abundance in the sample. Below is the equation used to convert cells per well to cells per mL.

$$Cells\ per\ mL\ in\ sample = \left(\frac{cells}{well}\right) \times \frac{well}{0.25\ mL\ lysate} \times \left(\frac{filter}{X\ mL\ sample}\right) \times \frac{Y\ mL\ lysate}{filter}$$

Here is an example of how you use the above equation to estimate target abundance in a sample. You filter 500mL (X) of water onto a filter and lyse this sample in 2.0 mL of lysis buffer (Y). Using the SHA system you obtain an O.D. that translates to 625 cells per well (from the dose response curve). Then using the formula you estimate a target abundance of 10 cells per mL in your sample.

$$\left(\frac{625\ cells}{well}\right) \times \frac{well}{0.25\ mL lysate} \times \left(\frac{filter}{500\ mL\ sample}\right) \times \frac{2\ mL\ lysate}{filter} = \frac{10\ cells}{mL\ of\ sample}$$

Controls

Positive and negative controls are available to check system integrity and performance (e.g. Greenfield *et al.* 2006).

Sample Collection and Preservation

Samples should be run or archived as soon as possible after collection. Prior to filtration, samples should be kept cool and protected from excessive light. If the sample cannot be run within several hours it should be filtered and stored frozen. A sample stored in liquid nitrogen can be held for up to 1 year or longer. Samples can also be stored in a -80 ºC freezer or on dry ice for up to 1 week. It may be possible to store preserved samples at room temperature as well (see Tyrrell *et al.* 2002).

Discussion and system considerations

The goal of SHA system is to give the research/monitoring community a method to quickly and conveniently screen samples for a variety of HAB species. Once samples are collected, processing takes approximately 1.25 hours. No target amplification is required so problems that can affect amplification based systems (e.g. extensive sample handling, PCR inhibition) are avoided. The absolute detection level of the SHA system is dependant on the designs of the capture and signal probes. An important feature of the SHA system is that it is relatively insensitive to biomass, so techniques such as sieving to collect large volumes of sample can be used without impacting system performance, provided proper control experiments have been performed to verify assay results from a given region and wide range of samples. Further increases in sensitivity can also be achieved by lysing the sample in a smaller volume of lysis buffer to increase target cell concentration. The SHA system is not suitable for very rare targets (e.g. single copy genes); in those cases some kind of amplification technique may be desirable.

All methods relying on molecular probes for detection can be subject to cross-reaction with non-target species. Therefore after an initial positive result, an alternative method (microscopy, toxin detection, PCR, etc.) should be used to confirm results until such time that confidence in the efficacy of probe is known. Some probes may work well for certain species in certain regions, but not all probes will work equally well in different geographic regions. Moreover, species designations as defined using traditional criteria (morphology, ultrastructure, pigments, etc.) may not agree with those based on rRNA sequence identity (e.g. see Scholin *et al.* 2004, Ayers *et al.* 2005, Lundholm *et al.* 2006). Provisions should also be made to store replicate samples in case of system failure or if results require further analysis or reconfirmation is desired.

Use of the SHA system requires that probes be available for the target species of interest. Currently, probes are available for a variety of HAB spp. (see above) as well as other organisms. The creation of probes for this system often requires iterative probe design (trial and error) which can incur considerable time and expense. Ideally, cultures of the targeted species are used to create calibration curves and to spot check the system when reagent batches are changed.

The SHA system uses chemistry that is designed to work at 30 ºC. If the ambient temperature exceeds this value, the system will not function properly and steps to lower ambient temperature will need to be taken. Some of the reagents used in the SHA system are required to be refrigerated and protected from direct sunlight.

Acknowledgements

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Appendix

Table 1. Equipment and suppliers. Note that some pieces of equipment, such as the plate reader, sample filtration system, heating block and refrigerator, are available as other models from a variety of vendors. Prices quote obtained December, 2007.

Equipment	**Supplier**	**Cat. Number**	**€**	**US $**
96-well Microplate reader that can read wavelengths 650 nm and 450 nm	Fisher Scientific	14-386-27	3821	5510
Optical filter 650nm	Fisher Scientific	14-386-59	196	283
Vacuum filter manifold	Fisher Scientific	09-753-39A	540	779
25 mm polysulfone filter funnel (250 mL) need 6, costs in total	Pall	4203	504	726
Vacuum pump	GAST	DOA P704 AA	303	443
Heating block	Fisher Scientific	11-716-68Q	223	322
12-Channel Pipettor 20-300 µL	Rainin	L12-300	412	595
Single Channel Pipettor 10-1000 µL	Rainin	PR-1000	184	265
Robotic processor	Saigene Inc[5].	6000-01	5708	8500
Refrigerator (4-8^{o}C)	Maytag	MBB1952HE	577	850
Cryogenic Storage vessel	Fisher Scientific	11-676-1C	2350	3393
Sum approx.			**14818**	**21666**

Table 2. Expendable reagents and supplies required for application of the SHA and relevant suppliers.

Material	**Supplier**	**Cat. Number**	**€**	**US $**
[1]Custom Plate One Probe Set; minimum order of 50 plates	Saigene[5]	call for order	25	38 per plate
[1]Custom Plate Two Probe Set; minimum order of 50 plates	Saigene[5]	call for order	27	40
[1]Custom Plate Three Probe Set; minimum order of 50 plates	Saigene[5]	call for order	28	42
[1]Custom Plate Four Probe Set; minimum order of 50 plates	Saigene[5]	call for order	29	44
[2]Assay Development Kit; minimum order of 25 plates	Saigene[5]	call for order	24	36
[3]Bulk lysis buffer, 500 mL	Saigene[5]	call for order	27	40
[4]Defined Kits (routine production), incl lysis buffer, prong, sample filters, etc.			Call for quote	Call for quote
25 mm Durapore filter (100 count)	Millipore	DVPP02500	60	86
13 mm syringe filter (100 count)	Millipore	SLHVT13NL	138	197
Polypropylene 12X75mm tubes (5000 count)	Fisher Scientific	14-961-11	318	459
5 cc Syringe (400 count)	Fisher Scientific	14-823-35	47	68
2 mL Cryovial (250 vials)	Fisher Scientific	03-337-7H	127	184
Filling boats (200 count)	Fisher Scientific	07-200-127	76	110
10% H_2SO_4 (dilute stock)	Fisher Scientific	A300-500	40	58
Sum approx.			**966**	**1402**

[1]Plates made custom to user specification. Saigene will fill and seal plates; user supplies capture and signal probes. Plates are configured with one to four probe sets. Price includes prongs; lysis buffer sold separately. Discount available for orders >50 plates.

[2]Plates filled with all reagents except capture and signal probes (to facilitate assay development). Price includes prong, buffers for preparing capture and signal probe solutions. Discount available for orders >25 plates.

[3]In addition to lysis buffer, Saigene can provide bulk quantities of other reagents used in the SHA; prices available on request.

[4]Defined Kits are those prepared entirely by Saigene. They differ from Custom Plate configurations in that Saigene provides user-defined probes, or those available through published articles. Defined Kits also include prongs and lysis buffer (volume based on the intended use of the plates), and can be bundled with sample and lysate filters if desired. The most likely application of Defined Kits are for research/monitoring programs where there is a defined set of target species/probes, sample and lysis volumes are fixed within well specified range, and users prefer not to take responsibility for procurement and quality assurance of probe stocks. Depending on the number of plates ordered, Defined Kits will generally exceed those in cost of the equivalent Custom Plate by 10-20% depending on probe costs and number of probe sets required per plate.

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13 Quantitative PCR for detection and enumeration of phytoplankton

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Introduction

Quantitative Polyemerase Chain Reaction (QPCR) is an extremely sensitive method which has been applied in recent years to detect and quantify different phytoplankton species in environmental samples (Bowers *et al.* 2000, Popels *et al.* 2003, Galluzzi *et al.* 2004, Coyne *et al.* 2005, Park *et al.* 2007, Touzet *et al*. 2009). Its application could revolutionise the study of microalgal population dynamics in marine systems as it allows the concurrent identification, enumeration and determination of viability of target species (Coyne and Cary 2005, Kamikawa *et al.* 2005). QPCR can be used on both seawater and sediment samples. The method is based on the amplification of specific DNA sequences. In assays developed to date for phytoplankton these sequances consist of ribosomal RNA (rRNA) genes. Many protocols have been optimised for the QPCR approach. Here, we describe an optimised protocol, which is based on the use of SYBR Green (a dye which specifically binds to DNA) and relies on a standard curve constructed with a DNA plasmid containing the cloned target sequence for quantification.

Basic Principles of Quantitative PCR (QPCR)

Polymerase Chain Reaction is a technique used to amplify *in vitro* a target sequence of DNA. The PCR is performed by heating and cooling an initial reaction mixture in a defined series of temperature steps. The reaction mix contains DNA from the sample to be tested, two different primers (small bits of artificially synthesised DNA complementary to the target DNA in which you are interested) nucleotides consisting of the four different bases required to make DNA, a DNA polymerase enzyme which will build DNA using the nucleotides and buffer containing various salts which are optimal for the functioning of the DNA polymerase. The different temperature steps are necessary to separate the two strands in the double helix DNA (denaturation step), to allow the binding of the primers to the complementary DNA present in the sample (annealing step) and to permit DNA synthesis by the DNA polymerase (elongation step). The specificity of the PCR is mainly due to the primer sequences which must be complementary only to the DNA region targeted for amplification. The annealing temperature is of particular importance. If too low an annealing temperature is used, then the primers may anneal to regions of DNA which are similar but not identical.

As the PCR progresses, the DNA generated by the reaction is used as a template for replication leading to the exponential amplification of the target DNA sequence. A typical PCR amplification profile consists of an exponential amplification of the target sequence, followed by a linear or plateau phase as reagents become exhausted as seen in Figure 1.

The qualitative analysis of the PCR reactions is performed at the end point of the reaction. This is usually performed by agarose gel electrophoresis and ethidium bromide staining. This method of visualising this PCR DNA product involves placing the PCR product in a well in a thin block of agarose gel and passing an electric current, negative to positive, through the gel. DNA is a negatively charged particle and will migrate with the current through the gel. Because all of the PCR products are the same size i.e. the size determined by the distance between where the two primers originally bound to the opposite strands of the DNA during PCR amplification, all the PCR products will migrate at the same rate forming a dense band of DNA. The agarose is infused with ethidium bromide, a stain which binds to DNA. Ethidium bromide fluoresces under UV light. When the gel is viewed under UV light the PCR product can be easily seen as a fluorescent band where the ethidium bromide has concentrated in the DNA. The brightness of the PCR band is related to the amount of PCR product present at the end of the PCR reaction (the plateau phase).

In quantitative real-time PCR (QPCR), reactions are analysed during the initial exponential phase rather than at the end point. PCR product formation is monitored after each cycle in real time by measuring a fluorescence signal which is proportional to the amount of PCR product generated. This is performed using a camera incorporated within a real-time PCR machine. Software converts the data recorded by the camera and allows the visualization of the amplification curves on a computer screen. An example of this can be seen in Fig. 2. The fluorescence can be generated by using intercalating (binding between the grooves of the double helix

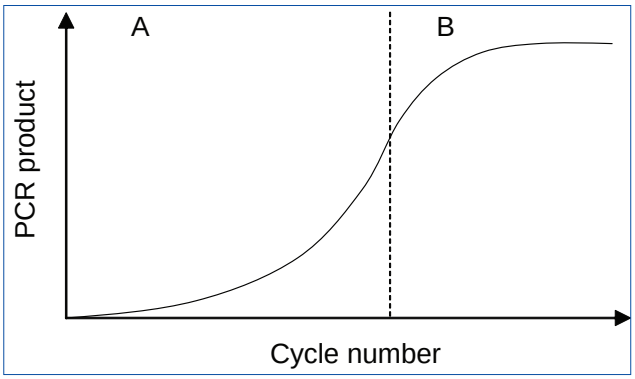

Figure 1. General PCR amplification profile. The exponential amplification phase (a) is followed by a linear or plateau phase (b), due to reactants exhaustion

The fundamentals of

The quantitative PCR method

Scope
Detection and quantification of target phytoplankton species.

Detection range
Detection performance varies with the sample volume, more precisely: 10 target sequences in a 25 µL reaction tube. The method is sufficiently sensitive to detect one cell.

Advantages
Allows identification of target phytoplankton to species level. Highly sensitive. No taxonomic expertise needed.

Drawbacks
Probes are only available for a limited number of target species. Specificity of probes must be established on a regional basis. Only one species or strain at a time can be analysed in a quantitative manner, unless a multiplex reaction is performed; equipment is still expensive.

Type of training needed
Instruction in setting up this technique should come from a person with an in-depth knowledge and experience of molecular biology particularly in real-time PCR (RT-PCR). A skilled molecular biologist should be available to solve any problems that may arise using this method.

Essential Equipment
Filtration apparatus/centrifuge, hybridisation oven, real-time PCR instrument.

Equipment cost*
Total set-up cost = €49426 ($69890)
See Appendix, Table 1 for details

Consumables, cost per sample**
€4.10 ($6.00 US) using a Millipore filter to collect cells or €3.00 ($4.40 US) using a microcentriguge to collect cells

Processing time per sample before analysis
Approximately 4 hours to process the sample.

Analysis time per sample
Approximately 3 hours to assemble and perform the RT- PCR. Up to 10 samples can be processed roughly in the same amount of time. Analysis of the real-time PCR results and calculation of cell concentration may require 15 minutes.

Sample throughput per person per day
A trained person can process up to 16 samples per day.

No. of samples processed in parallel
Up to 16 samples.

Health and Safety issues
Relevant health and safety procedures must be followed. SYBR Green is a DNA intercalating dye.

*service contracts not included
**salaries not included

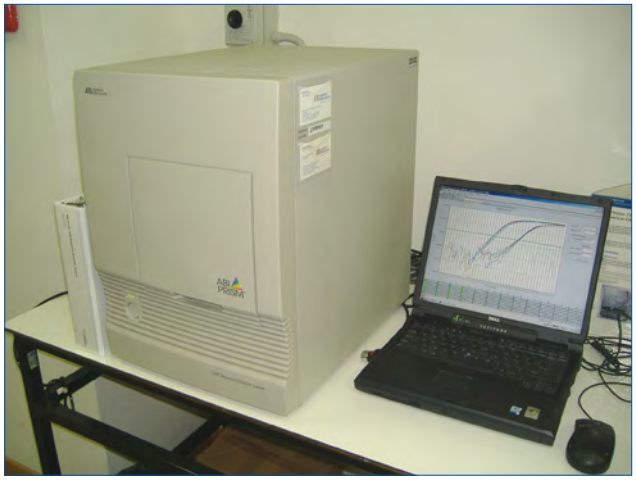

Figure 2. Real-time PCR instrument ABI PRISM 7000 SDS (Applied Biosystems).

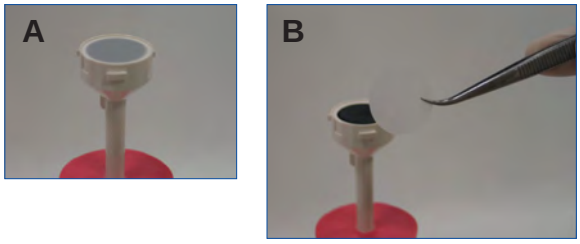

Figure 3. Filter system (A) with 3 µm Millipore TSTP membrane (B).

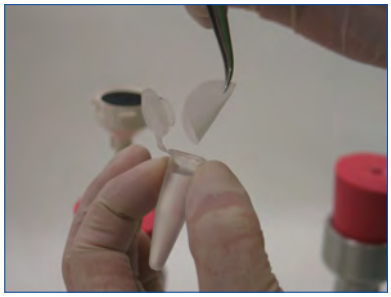

Figure 4. Collecting cells from the filter with 1 mL artificial sterile seawater in a 1.5 mL tube .

Figure 5. Cell lysis by sonication.

DNA) fluorescent dyes or a number of alternative real-time PCR chemistries which use probes (short synthetically made pieces of DNA) which fluoresce when bound to their complementary target DNA following PCR amplification (e.g. Hydrolysis probes (TaqMan), Hybridisation probes (FRET).

For each sample, the fluorescence signal of the reporter dye (i.e. the dye which fluoresces in proportion to the amount of PCR product produced) (e.g. SYBR) is divided by the fluorescence signal of the passive reference dye (ROX) to obtain a ratio defined as the normalised reporter signal (Rn). ROX is a dye present in the reaction mix which gives out a standard level of fluorescence independent of PCR amplification. It is used to normalise for differences in amount of reaction mix due to pipetting errors or evaporation. Some QPCR machines do not require the use of ROX. The higher the starting amount of the target molecule, the earlier a significant increase in fluorescence (Rn value) is observed. The parameter Ct (threshold cycle) is defined as the fractional cycle number at which the fluorescence crosses a fixed threshold above the baseline. The amount of target sequence in an unknown sample is calculated by plotting the Ct value on the standard curve. The standard curve is generated by the QPCR instrument software by plotting Ct values versus the log of initial target concentration, and by performing a linear regression. The PCR efficiency can be calculated from the slope of the line using the equation:

$$\text{Efficiency} = 10^{(-1/\text{slope})} - 1$$

If the PCR has 100% efficiency, the amount of PCR product will double after each cycle and the slope of the standard curve will be -3.33 (i.e. 3.33 cycles gives a 10 fold increase/decrease between the 10 fold serial dilutions used to generate the curves). A PCR efficiency of at least 90% (slope = 3.6) is generally required for reliable quantitative results.

Materials

Laboratory facilities

Laboratory facilities necessary for quantitative analysis of phytoplankton by PCR are consistent with those found in a standard molecular biology laboratory equipped for DNA cloning, purification, quantification and real-time (quantitative) amplification.

Equipment, Chemicals and Consumables

The equipment, chemicals and consumables used in this method are presented in the Appendix, at the end of this chapter (Tables 1-2). Suppliers, Catalogue numbers and estimated cost in Euros and US Dollars are also listed in Tables 1-2.

Method

Sample processing (culture or seawater samples fixed with acidified Lugol's solution)

Microalgal cultures or seawater samples fixed with acidified Lugol's iodine solution (see chapter 2 for recipe) have to be collected and lysed appropriately in order to generate lysates

(or starting material) suitable for use in QPCR. In many instances the sample will need to be concentrated prior to starting the QPCR method. This can be achieved by either centrifugation or filtration.

Collection of Phytoplankton Cells Using a Centrifuge

1 Spin phytoplankton cells at 3000 rpm for 15 minutes at 12 ± 1 ºC and remove the supernatant carefully. This removes the acidified Lugol's fixative. A swinging bucket centrifuge is required to allow for the generation of a concentrated pellet.
2 Resuspend cells in a suitable volume of artificial sterile seawater and transfer to 1.5 mL tube.
3 Spin at 3000 rpm for 15 minutes at 12 ±1 ºC in a swinging bucket rotor and remove the supernatant (leaving approximately 100 µL).
4 Spin again at 10000 rpm for 10 seconds at 12 ± 1 ºC and remove the remaining supernatant leaving the pellet in the tube.

Collection of Phytoplankton Cells Using a Filter System

1 Filter the appropriate volume of sample to be processed onto a 3.0 µm Millipore TSTP membrane (Fig. 3).
2 Wash the filter with 1.0 mL of artificial sterile seawater in a 1.5 mL tube (Fig. 4). This collects the cells from the filter surface.
3 Spin the cells at 6800 rpm for 5 minutes in a microcentrifuge and remove the supernatant (leaving approximately 100 µL).
4 Spin at 10000 rpm for 10 seconds and remove the remaining supernatant leaving the pellet dry.

Cell Lysates Preparation

1 Freeze pellet at -80 ± 1 ºC for 15 minutes.
2 Resuspend the frozen pellet with 400-600 µL lysis buffer (PCR buffer 1X, NP40 0.5%, Tween 20 0.5%, proteinase K 0.1 mg mL^{-1}) at a concentration of 1 x 10^5 – 2 x 10^5 cells mL^{-1}.
3 Sonicate twice at 50 W for 10 seconds and incubate at 55 ± 1 ºC for 2-4 hours (Figs 5-6), vortexing at intervals of 30 minutes.
4 Boil for 5 minutes and centrifuge at 12000 rpm for 2 minutes. This will eliminate cell debris and impurities.
5 Transfer the supernatant to a clean microcentrifuge tube and store this sample lysate at -80 ± 1 ºC for 2-3 days or process it immediately.

Standard Curve Preparation

Plasmid generation

A PCR product, generated as described previously, which contains the complementary target DNA sequence to which the QPCR primers bind (and probe if a probe based chemistry is used) is enzymatically joined at both ends (ligated) to a specific double stranded DNA sequence called a plasmid. This forms a circular DNA construct. A plasmid is DNA, usually in circular form, which is capable of replicating itself independently of cell replication. A single plasmid containing the target sequence is introduced into a bacterial cell following a chemical or electrical reaction. The bacteria is placed on a nutritious gel and multiplies to form a visible colony consisting of thousands of bacterial cells. The plasmid is passed to each new cell following cell division. Within each cell, the plasmid will also replicate many times, and with it the original PCR product. The plasmids are purified from the bacteria resulting in a highly concentrated source of pure PCR product containing the target sequence for the QPCR assay. There are many types of plasmids and bacterial cells commercially available and numerous methods for purification, again many of which are incorporated into commercially available kits.

The standard curve can be constructed with 10-fold serial dilutions of a plasmid containing the target sequence. Usually, the curve range extends from 1.0 × 10^2 to 1.0 × 10^6 plasmid copies but it can be adjusted according to the assay detection range required. The plasmid copy number is calculated using the following formula:

$$\text{Molecules } \mu L^{-1} = (A \times 6.022 \times 10^{23})/(660 \times B)$$

where: A = plasmid concentration (g μL^{-1}); B = length of the plasmid containing the cloned sequence; 6.022 × 10^{23} =

Table 1. Master mix preparation for real-time PCR.

Reagent	Amount per reaction	Final concentration
dH_2O	variable	
Commercial mix containing SYBR Green 2X	12.5 µL	1X
*$MgCl_2$ 25 mM	1-5 µL	1-5 mM
Forward primer 10 mM	0.25-1.5 µL	100-600 nM
Reverse primer 10 mM	0.25-1.5 µL	100-600 nM
*DNA Polymerase 5U/mL	0.125-0.25 µL	0.025-0.05 U/mL
Diluted cell lysate	1-2 µL	-
Final volume	**25 µL**	

**Only if required. Some commercial mixes (i.e. Applied Biosystems) already contain $MgCl_2$ and DNA polymerase*

Figure 6. Samples in a thermal block at 55 °C.

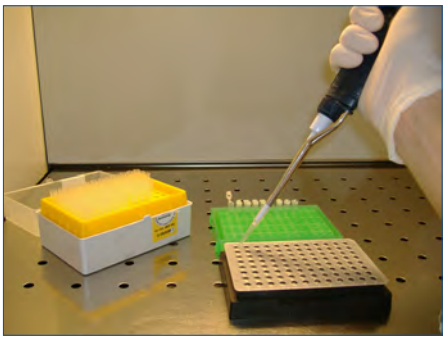

Figure 7. Dispersing the reaction mixtures in the optical plate (25 µL each well).

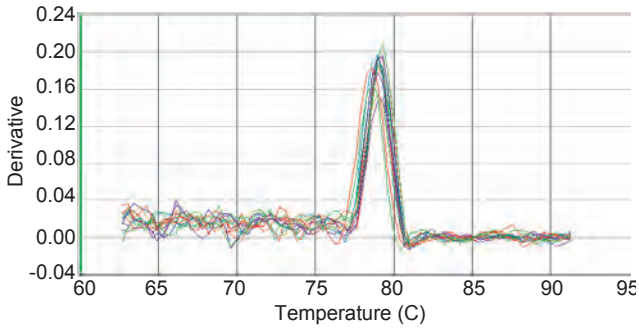

Figure 8. Example of dissociation protocol results. The graph shows the derivative of fluorescence emission variation plotted against the temperature increment. The peak corresponds to a specific 173-bp PCR product. No aspecific products or primer dimers are visible.

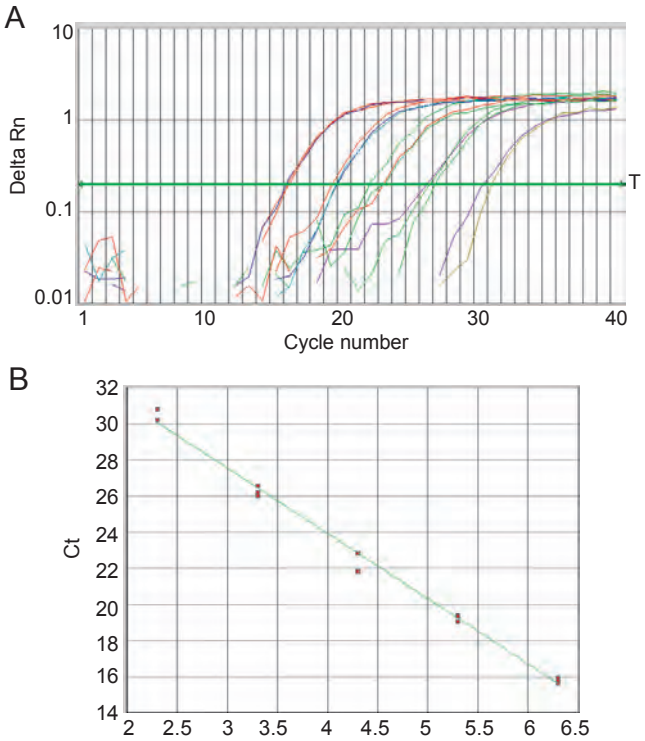

Figure 9. Example of standard curve obtained with a plasmid containing 28S rDNA sequence of *Alexandrium fundyense.* (A) Amplification plots with plasmid copy number from 2.0 X 10^6 to 2.0 X 10^2. The cycle number is plotted vs the Delta Rn. The Delta Rn represents the normalized reporter signal (Rn) minus the baseline signal established in the early PCR cycles. Three replicates were performed for each reference DNA sample. T: threshold. (B) Calibration curve plotting log starting copy number vs Ct. Slope: –3.61; correlation coefficient (R^2): 0.9939.

Avogadro's number; 660 = average molecular weight of one base pair. The accurate determination of the plasmid concentration can be performed using a spectrophotometer or a fluorimeter. Once quantified, it is recommended to store the plasmid at -80 ºC in small aliquots to avoid repeated freeze/thaw cycles. Moreover, plasmid dilutions should be freshly prepared for each PCR run.

Dilutions of Sample Lysates and Plasmid

1 Make scalar dilutions 1:10 of the sample lysates with PCR-grade H_2O.
2 Make scalar dilutions 1:10 of the plasmid with PCR-grade H_2O (from 1.0×10^6 to 1.0×10^2).

Quantitative PCR Assay

1 In a separate clean room set up the QPCR master mix described in the Table 1. Make triplicate PCR tubes for each lysate and plasmid dilution (see above). This is required to determine the intra-assay variability of results. For this purpose, it is practical to aliquot a reaction volume equivalent to 3 reactions (75 µL) in one standard PCR tube, add the template, and then disperse the reactions in optical tubes/plates (25 µL per tube/well) (Fig. 7). Quantitative PCR assays are performed in a final volume of 25 µL using the SYBR Green chemistry as presented in Table 1. To reduce cost, it is possible to set up QPCR mixtures considering a final volume of 12.5 µl instead of 25 µl. In this case, particular care should be taken to avoid volume differences between tubes.
2 Perform the QPCR in a real-time PCR instrument under the following reaction conditions: 95 ºC for 5 minutes; 40 cycles of 15 sec at 95 ºC, 1 min at 60 ºC, with a final dissociation protocol to ensure the absence of non-specific PCR products or primer dimers (Fig. 8). These conditions can be modified depending on the commercial mix used and the characteristics of each primer.

Due to the sensitivity of the PCR method, it is crucial to avoid contamination. It is therefore necessary to set up the reactions in a clean area (i.e. in a PCR cabinet), free of potential plasmid or PCR product contamination. Always include one or more negative controls (blank sample with no target template).

PCR efficiency is important for quantification purposes: to maximize efficiency, it is advisable to design primers producing a PCR product no more than 100 bp long. These short amplicons can also allow the use of partially degraded DNA, without loss of quantification performance. It is also necessary to include the standard curve in each PCR run, due to the possible variability from one PCR to another.

To avoid variability and low yields in DNA purifications, crude cell lysates can be used as the DNA template in the PCR reactions. These lysates contain components that can interfere with PCR reactions and it is therefore necessary to find a template amount which can be amplified without any inhibitory effect. For this purpose, the QPCR assay is performed, at least during the assay optimisation, with 10-fold serial diluted lysates until quantification results become proportional to sample dilutions. Usually, dilutions from 1:10 to 1:1000 are sufficient to establish the correct conditions. It is important to note that this approach would be possible when the target sequence is present in high copy number in the cell (e.g. rRNA genes), otherwise the sensitivity of the method will drop significantly.

Analysis of Results

The analysis of the results is performed using the specific QPCR machine software.

1 Set a suitable baseline and threshold value, if the instrument does not do it automatically. Plasmid amplification plots and a standard curve similar to the one presented in Fig. 9, should appear.
2 Using experimental results, the number of target DNA copies per µL of the original sample can be caluclated

from the standard curve.

3 In order to estimate the total number of cells in the initial sample, the number of target DNA copies per µL in the sample is divided by the number of rDNA copies per cell. This is then multiplied by the initial lysate volume.

Formulas for Calculating Results:

$$N = [(A/B) \times d] \times (V/C)$$

N: Total number of cells in the initial sample
A: number of target DNA copies per PCR tube (calculated by instrument software)
B: µL of cell lysate in the PCR tube
C: target DNA copies per cell (to be determined for each target species using cultured cells)
d: lysate dilution factor
V: initial lysate Volume expressed in µL

Sample Preservation and Storage

Phytoplankton samples can be preserved with acidified Lugol's iodine solution and stored at 4°C in the dark. Experience has shown that reliable quantitative PCR results can be obtained from samples stored up to one year.

Discussion

The QPCR has proven to be a powerful method for the quantification and detection of phytoplankton species in environmental samples (Galluzzi *et al.* 2004, Hosoi-Tanabe and Sako 2005, Dyhrman *et al.* 2006).

The use of commercial PCR master mix containing intercalating dyes such as SYBR Green is a simple and inexpensive approach. The assays based on intercalating dyes are very sensitive, but it is noteworthy that any double stranded non target DNA can also be detected, leading to misinterpretation of results. For this reason, the specificity of the primer is crucial. An increase in the specificity of the assay can be obtained using a TaqMan probe but this may result in a loss of sensitivity.

Theoretically, this technique can be applied to any phytoplankton species with DNA sequence data available so specific primers or probes can be designed. However, some drawbacks need to be taken into account when applying QPCR method to phytoplankton. In particular, when a target sequence cloned into a plasmid is used as a standard, it is essential to know the amount of target DNA per cell, in order to determine the cell number in the field sample. This requires preliminary work with cultured strains to optimise the method for each target species/strain. Due to the possibility of variations of target gene copy number (particularly in case of rRNA genes) among different strains, the level of accuracy of the quantitative real-time PCR assays can be affected, as it has been shown for Mediterranean *Alexandrium* species (Galluzzi *et al.* in press). For this reason, the method should be tested and optimized with the local phytoplankton population in the geographical area to be investigated. Primer or probe specificity needs to be tested with the local population of phytoplankton to ensure absence of non-specific target amplification.

Compared to classical microscopy techniques, the main advantages of the QPCR in phytoplankton monitoring include specificity, sensitivity and applicability to preserved environmental samples. Sample preservation is often necessary, but the use of fixatives may cause the morphology distortion of some phytoplankton species, making it more difficult to distinguish them from closely related species using a microscope. The QPCR sample throughput can be up to 27 triplicate samples per 96-well plate, including the standard curve. This sample throughput may reduce working time compared to the microscope-based methods when a high number of samples need to be analysed. It is also noteworthy that the collection of phytoplankton using a filter based system is faster then the centrifugation method. This alternative speeds up the entire process thus reducing the handling time.

QPCR instrument prices are becoming affordable for small research groups and are now common in many molecular biology laboratories. The consumable cost per sample has been estimated in $4 - $6 depending on the method/master mix/chemistry used, making the QPCR an affordable method for monitoring purposes.

In QPCR assays, only one species or strain can be analysed at a time unless a multiplex reaction is performed. This usually requires the use of more expensive fluorescent probes (e.g. TaqMan probes) instead of intercalating dyes. Although multiplexing and/or multiprobing are powerful tools for molecular investigations of specific groups of toxic algae, their development and validation can be difficult and expensive (Handy *et al.* 2006).

This method can be still considered at the developmental stage. If this method is incorporated into future monitoring programs for HAB-species, a subset of samples could also be checked using a more traditional microscopic technique. This type of quality control would validate the presence of the target species on a selected subset of samples and highlight any problems that may arise with the method.

Acknowledgements
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Appendix

Table 1. Equipment and suppliers.

Equipment	**Supplier**	**Cat. Number**	**€**	**US $**
Real-time PCR instrument ABI PRISM 7300 SDS	Applied Biosystems	4351101	29840	42099
Ultrasonic Homogeniser Branson S-150D	VWR international	33995	2110	2980
Analog Dry Block Heater	VWR international	12621-110	344	485
Swing-bucket Centrifuge IEC-CL30	Thermo Scientific	11210904	5532	7856
Mini-Centrifuge Heraeus ”Biofuge Fresco”	Thermo Scientific	75002420	3600	5110
Spectrophotometer	Shimadzu, Japan	UV-2401 PC	8000	11360
Sum approx.			**49426**	**69890**

Table 2. Chemicals and suppliers.

Chemicals/consumables	**Supplier**	**Cat. Number**	**€**	**US $**
Isopore membrane filter 3.0 µm TSTP	Millipore, USA	TSTP02500	129	183
Lugol solution (1L)	Sigma-Aldrich Srl, Italy	62650	26	37
*SYBR Green PCR Master Mix, 2 X 200 reactions	Applied Biosystems, USA	4364344	664	936
MicroAmp Optical 96-well Reaction Plate, 20/pkg	Applied Biosystems, USA	4306737	152	214
Optical Adhesive Covers, 25/pkg	Applied Biosystems, USA	4360954	76	107
Tween 20	Sigma-Aldrich Srl, Italy	P1379-100ML	26.9	38
NP 40	Sigma-Aldrich Srl, Italy	74385-1L	36	50
Proteinase k	Sigma-Aldrich Srl, Italy	P6556-25MG	39.1	55
Primers forward and reverse (25 nmol)	Invitrogen, Italy	-	13	18
Tubes 1.5 mL	Sarstedt, Germany	72690 - 500/pack	16	22
Polypropylene conical tubes 50 mL	BD Biosciences	352070 - 500/case	99	140

*Different commercial SYBR Green mixtures can also be used (for example Hot-Rescue Real Time PCR KIT-SG from Diatheva, Italy).

14 Tyramide signal amplification in combination with fluorescence *in situ* hybridisation

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Introduction

Tyramide Signal Amplification (TSA) - Fluorescence *in situ* Hybridisation (FISH) provides an enhanced fluorescence signal from molecular probes The oligonucleotide probe is labelled with the enzyme horseradish peroxidase (HRP). After hybridisation the HRP catalyses the deposition of a phenolic compound, a FITC (fluorescein isothiocyanate) labelled tyramide. In the presence of hydrogene peroxide, the immobilised tyramide binds to electron rich moieties (mostly tyrosine residues) of adjacent proteins. This TSA reaction results in a signal enhancement of up to 30 times more intensity then traditional labelled fluorescent probes (Fig. 1).

Solid phase cytometry

Solid phase cytometry (SPC) combines the advantages of flow cytometry with image analysis (Kamentsky 2001), and enables the fast detection and enumeration of micro-organisms down to one cell in a sample (Lemarchand *et al.* 2001). In SPC, the laser is moved over cells immobilised onto a solid support, which allows the rapid enumeration of several thousand cells with a similar accuracy to flow cytometry (Darynkiewicz *et al.* 2001). The ChemScan™ instrument (Chemunex, France) is a SPC, which was initially developed for industrial and environmental microbiology (Mignon-Godefroy *et al.* 1997, Reynolds and Fricker 1999). It was adapted for the detection of toxic microalgae using antibody (West *et al.* 2006) and oligonucleotides probes (Töbe *et al.* 2006). The ChemScan™ uses a 488 nm argon-ion laser and is therefore suited for probes or tyramides labelled with FITC. Algal cells are collected by filtration onto a polycarbonate membrane, hybridised, and subsequently scanned (Fig. 2). Fluorescent events are detected and a computer applies various criteria to discriminate between "true" and "false" signals deriving from hybridised cells using the MatLab-based software (Matworks, Natick, USA) for comparison with Gaussian curves (Roubin *et al.* 2002). The exact positions of positively identified cells are shown as coloured spots on a display of the membrane in a scan map. The hybridised cells can be visualised by transferring the membrane with its membrane holder to an epifluorescence microscope equipped with a computer controlled motorised stage that is connected to the ChemScan™. This allows each positive data point to be visually validated by microscopic examination immediately after scanning as true positives or false positives (Reynolds and Fricker 1999, Roubin *et al.* 2002). This method is very fast, positive hybridised cells are counted within 3 minutes and no positive hybridised cell can be overlooked by the operator as in standard FISH applications.

TSA-FISH is required for reliable automated detection of target cells with the ChemScan™ to increase the peak fluorescence intensity as a discrimination pattern in the computer software. Since TSA-FISH labelled cells reach very high fluorescence intensities, this allows the computer software to develop discrimination patterns between labelled and non-labelled cells. This new automated method for counting microalgae is, however, only adequate for round and spherical cells at the moment and not for long colony forming species, like the diatoms *Pseudo-nitzschia*. The computer software presently used must be revised in order to count filamentous colony forming microalgae. Microscopic verification of positive cells is recommended. This can be performed after confirming that the FISH labelling has been successfully completed.

Presently, the application of only one single probe label is possible, because of the single laser of the ChemScan™ and it is therefore not possible to detect more than one species on a filter at a time. The effectiveness of a new probe label is, however, under development. This new probe label is excited with the present ChemScan™ laser, like standard FITC-labelled cells, but it has a different emission wavelength and with the installation of a suited Photomultiplier, two different probes could be applied.

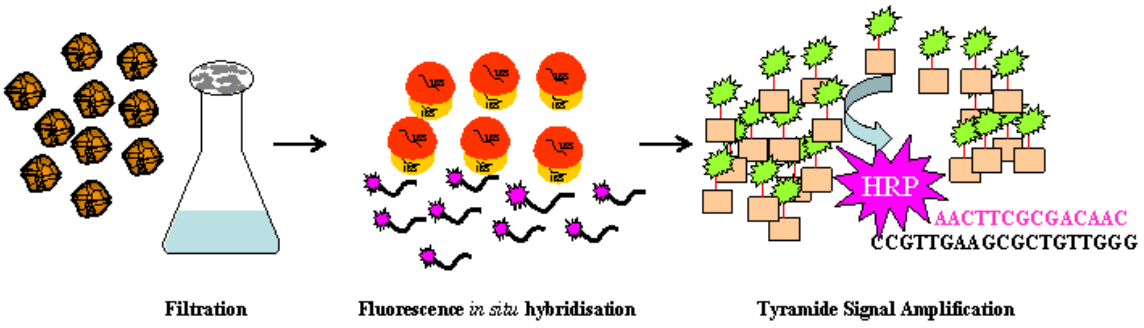

Figure 1. Tyramide signal amplification.

The fundamentals of

The tyramide signal amplification - fluoresence *in situ* hybridisation in combination with solid phase cytometry method

Scope
Semi-automated detection and quantification of target phytoplankton species.

Detection range
Detection of microalgal RNA by FISH is very sensitive. The number of cells that can be detected depend on the sample volume. High biomass can obscure the view of target cells.

Advantages
Simple and easy to use. Sample volumes can easily be adjusted. Simultaneous labeling and detection of multiple species is possible. Strong yellowish/green labelling of target cells minimises confusion with non-target cells. Semi-automated analysis.

Drawbacks
Probes are only available for a limited number of target species. Rigorous optimisation and specificity testing on local strains is required before the method can be implemented. Finite storage time for samples. Complex sample processing procedure may result in cell loss. At present very high start-up costs. Intensity of positive reaction may vary with cell conditions. Access to molecular expertise is essential. Appropriate laboratory facilities for storage and processing of probes and reagents are necessary.

Type of training needed
Instruction in setting up this technique should come from a person with an in-depth knowledge and experience of molecular biology. Approximately one week of supervised training required to properly perform the method.

Essential Equipment
Solid phase cytometer and epifluorescence microscope with automated stage; filtration unit, vacuum pump, hybridisation oven.

*Equipment cost**
Total set-up cost = €183310, $ 269.795
See Appendix, Table 1 for details

*Consumables, cost per sample***
€11 ($16.13), see also Appendix, Table 2

Processing time per sample before analysis
5-30 minutes. Filtering time depends on the amount of phytoplankton in the samples.

Analysis time per sample
The TSA-FISH procedure and analysis with SPC requires 4.25 hours of handling time per filter of 3 minutes for scanning and 2 minutes for verification of the results.

Sample throughput per person per day
A trained person can process up to 36 samples per day depending on the number of target organisms in each sample.

No. of samples processed in parallel
36

Health and Safety issues
Relevant health and safety procedures must be followed. The following chemical is particularly hazardous: Formamide.

*service contracts not included
**salaries not included

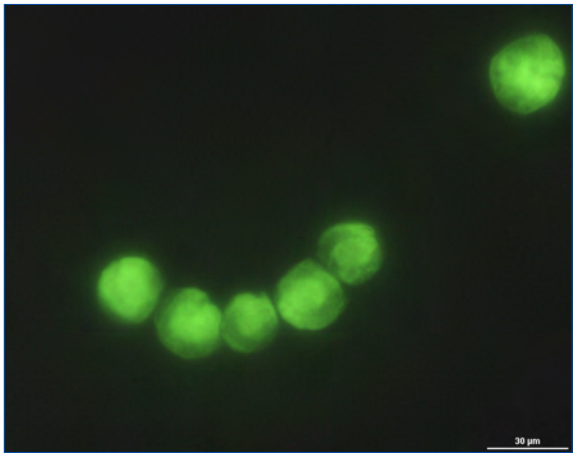

Figure 2. Tyramide Signal Amplification FISH with *Alexandrium fundyense* cells and HRP-labelled probe ATNA02 (John *et al.* 2005), photographed at 40x objective lens.

Materials

Laboratory facilities

Molecular biology laboratory

Required Equipment (essential)

The quantitative TSA-FISH in combination with solid phase cytometry requires the following equipment:

- Solid phase cytometer
- Epifluorescence microscope with motorised stage
- Filter vacuum manifold or glass filter equipment
- Hybridisation oven
- Vacuum pump
- Pipettes 1-20 µL, 100-100 µL + sterile tips, 1-50 mL pipette + sterile tips
- Autoclaved glassware
- Disposable gloves, tweezers,
- White polycarbonate filter membranes: 25 mm diameter, pore size depending on cell size
- Support pads

Chemicals and Consumables

Solutions for Fixation

Saline ethanol (Scholin *et al.* 1996), prepared freshly for each experiment because of the formation of precipitates

25 vol. 95 % ethanol
2 vol. Milli-Q water
3 vol. 25X SET

or modified saline ethanol (Miller and Scholin 2000) stable at room temperature for several months without precipitate formation

22 vol. 95 % ethanol
5 vol. Milli-Q water
3 vol. 25X SET buffer

25X SET

3.75 M NaCl
0.5 M Tris/HCl
25 mM EDTA
pH 7.8, filter sterilised

Solutions for Fluorescence In situ Hybridisation

Hybridisation buffer

0.1 % (v/v) Nonidet-P40 (Sigma N-6507)
x % (v/v) Formamide
2 % blocking reagent (Roche, Mannheim, Germany)

Wash buffer:

1X SET

Solution for quenching endogenous peroxidase activity

3 % Hydrogene peroxide (H_2O_2) in filter sterilised deionised water

Solutions for amplification reaction

TNT-Buffer

0.1 M Tris-HCL, pH 7.5
0.15M NaCl
0.05 % Tween 20

Tyramide substrate solution

One volume of 40 % (wt/vol) dextrane sulfate (Sigma-Aldrich, Munich, Germany) in sterile deionised water, is mixed with one volume of amplification diluent and a 1:50 dilution of the fluorescein labelled tyramide (Perkin Elmer, Boston Ma, USA, diluted in Dimethylsulfoxide) in sterile deionised water and stored in the dark.

Solution for counterstaining

Citifluor/DAPI (4',6'-diamidino-2-phenylindoline) mixture (0.5 mL sterile deionised water, 1 mL Citifluor (Citifluor Ltd., Cambridge) and 1.5 µL DAPI solution (1 µg μL^{-1}, Invitrogen, Germany, in sterile deionised water)).

Probes

Horseradish peroxidase labelled probes purchased from Thermo Scientific, Germany are delivered lyophilized. The HRP-label is anchored at the 5'-end of the oligonucleotide. Stock solution of 1 µg μL^{-1}, and working solutions of 500 μL^{-1} and 50 ng μL^{-1} should be prepared in 1X TE buffer, pH 7.8- 8.0. Probe stock solution should be stored at -80°C and working solutions at -20°C. Since, HRP-labelled probes are not light sensitive it is not necessary to work in the dark. However, from the tyramide signal amplification step on working in the dark is necessary, because of the incorporated light sensitive FITC-labelled tyramide. More information on the equipment, chemicals and consumables used in this method are presented in the Appendix at the end of this chapter.

Method

Handling Time

Based on 10 samples processed in parallel

0.5 h	Set-up and filtering step
1 h	Fixation
0.25 h	Wash step and probe addition
1.5 h	Hybridisation and tyramide signal amplification
0.5 h	Analysis of 10 filter with solid phase cytometer
0.5 h	Clean up

TOTAL HOURS 4.25 h

Sample Preservation and Storage

- Filter 5 mL sample onto a polycarbonate filter with the lowest possible vacuum and incubate in the fixative for at least 1 hour at room temperature or overnight at 4 °C.
- Remove the fixative by filtrating and incubating for 5 minutes with hybridisation buffer without a probe.

Filters can be dried and stored for at least one month at room temperature or hybridised directly afterwards.

Quenching of Natural Occurring Peroxidases

- Quench naturally occurring endogenous peroxidase activity by treating the filters with 100 µL 3 % H_2O_2 per filter at room temperature for 15 to 30 minutes to avoid unspecific staining.
- Rinse the filter in sterile deionised water to remove excess H_2O_2.

Hybridisation

- Cover the filters with 80-100 µL hybridisation buffer containing the horse radish peroxidase labelled probe (final concentration of probe in hybridisation buffer: 5 ng μL^{-1}) and hybridise 1.5-2 hours at 37 °C.
- Stop the hybridisation by adding pre-warmed (37 °C) 1X SET wash buffer and then wash the filters with 1X SET for 10 minutes at 37 °C.

Tyramide Signal Amplification

- Equilibrate the filters for 15 minutes in TNT buffer at room temperature.
- Remove excess liquid by putting the filters on blotting paper, staining should be conducted before they are completely dry.
- Incubate each filter with 100 µL Tyramide substrate solution for approximately 30 minutes at room temperature in the dark.
- Rinse the filters in TNT-buffer and wash for 15 minutes at 55 °C in TNT-buffer. Then rinse the filter in sterile deionised water, air dry and store at -20 °C pending analysis by ChemScan™.

Optional Step

Counterstaining

- Counterstain the filters with a Citifluor/DAPI mixture for 10 minutes at room temperature. Wash with sterile deionised water for 1 minute and incubate in 80 % ethanol (v/v) for 30 seconds to remove an excess of staining solution. Air Dry and store at -20 °C or examine directly with the ChemScan™. Citifluor is used as an antifade and the blue DAPI nucleic acid stain effectively stains double stranded DNA and enables a general overview of all cells on a hybridised filter.

Formulas for Calculating Results

The entire filter is automatically counted by the solid phase cytometer and afterwards validated randomly by the operator. Hence the cell number is reflective of the amount of original sample that was processed and preserved as well as a the volume that was filtered for hybridisation.

To calculate cells L^{-1}:

$$Cell\ concentration\ (cells\,L^{-1}) = \left(\frac{Positive\ cell\ count\ on\ whole\ filter\ (N)}{Volume\ of\ sample\ \ (mL)}\right) * 1000$$

where N is the number of positive cells on the whole filter and V (mL) is the volume of sample used.

Analysis of hybridised filters with SPC in combination with epifluorescence microscopy

The ChemScan™ system (Fig. 3) must be calibrated on a daily basis with a standardised amount of FITC labelled latex beads, diameter 2-3 µm (Standard C, Chemunex, France), in 100 µL. In order to verify that the laser is working properly, the number of fluorescence signals recorded by the ChemScan™ must be cross referenced with the number of counted latex beads in solution.

1. The 100 µL solution with the latex beads is filtered onto a black polycarbonate membrane (25 mm diameter, 0.2 µm pore size, Chemunex, France). To support the filter membrane, a black support pad is mounted by applying 100 µL ChemSol B16 (Chemunex, France) to a membrane holder. Then the filter membrane with latex beads is laid onto the support pad. The filter is scanned with the application C control.
2. For TSA-FISH filter also a black support pad is mounted by applying 100 µL ChemSol B16 (Chemunex, France), overlaid by the hybridised filter and the the application tvcbio1 (tvc: total viable counts) is used. The peak intensity is manually changed from 250 to 2500 to prevent the enumeration of false positive autofluoresing particles with lower peak fluorescence intensity.
3. Immediately after the scan, signals are validated using an epifluorescence microscope (Nikon, Eclipse E 800) equipped with filter blocks for FITC (Nikon Filter Block B-2A) and DAPI (Nikon Filter Block UV-2A) and with a motorised stage (Prior Scientific, UK). Images are captured with a digital camera (CCD-1300CB, Vosskühler, Germany) and analysed with the Nikon software Lucia G.

Discussion

Traditional FISH methods have limitations when counting samples with low target cell densities as well as in the number of samples that can be analysed per day. The time involved to count an environmental sample will vary with the diversity of the sample and the skill of the operator. For a high sample throughput, FISH has been combined with flow cytometry, which allows the analysis of different cell parameters. However, a combination of flow cytometry and microscopy of single detected fluorescent microalgae is difficult. In addition, because of the limited sensitivity at lower cell concentrations this method is not suited for the detection of cells at low concentrations in environmental samples. The solid phase

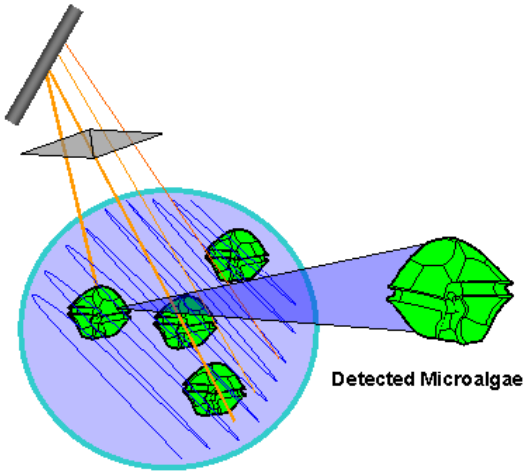

Figure 3. Overlapping scan with the ChemScan.

cytometer has the advantage of a direct combination of automated counting and epifluorescence microscopy, allowing the microscopically verification of each single cell detected. The solid phase cytometry in combination with TSA-FISH enables the efficient detection of a single cell in a filtered volume in less than 5 h. For a reliable automated detection of target cells with the ChemScan™, signal amplification is necessary. This method has great potential for application in analysing field material where a rapid and reliable detection and enumeration of target cells is required. The shape of the algae may cause problems, e.g., the ChemScan™ cannot count long chain forming cells like *Pseudo-nitzschia* cells. The adaptation of improved software could help to overcome this problem. Additionally, the actual high price of this machine and the additional costs of an epifluorescence microscope equipped with an automatic stage is a limiting factor.

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Appendix

Table 1. Equipment and suppliers.

Equipment	Supplier	Cat. Number	€	US $
Filter Manifold	Millipore, USA	XX2702550	400	587
Vacuum pump	Omnilab, Germany	9.881	391	574
Incubator ”Shake’n’Stack”	VWR, Germany	7996	2310	3387
Epifluorescence microscope, e.g. Nikon Eclipse E800 with motorized stage	Nikon, Japan	MAA600BA	29000	42521
Solid phase cytometer e.g. ChemScan™	Chemunex, France		152000	222870

Table 2. Chemicals and suppliers.

Chemicals	Supplier	Cat. Number	€	US $
Ethanol, absolute, 1L	Merck	1.009.832.500	73	107
Milli-Q water	Most labs have an in-house supply			
Sodium chloride, 1 kg	Sigma-Aldrich	S9888	23	34
Tris/HCl, 500 g	Sigma-Aldrich	T3253	96	141
Nonidet-P40, 100 mL	Roche	11754599	74	109
EDTA, 500 g	Sigma-Aldrich	E7889	56	82
Isopore white polycarbonate membrane filter, 3 µm pore size, Qty. 100	Millipore	TSTP02500	130	191
Hydrogene peroxide	Pharmacy	-	4	6
HRP-labelled probe, approx. 300 µg	Thermo Scientific	-	235	345
Deionized formamide, 100 mL	Sigma-Aldrich	F 9037	46	67

Appendix: Acronyms and Notation

Acronym	Meaning
ABD	Area-Based spherical Diameter
ADPA	N-Phenyl-1,4-benzenediamine hydrochloride
ALGADEC	Development of a RNA -Biosensor for the Detection of Toxic Algae
approx.	Approximately
ARB	“arbor ”=tree
BSA	Bovine Saline A
CCD	Charge Coupled Device
CEN	Comité Europeén De Normalisation
ChemScan™	Lazer scanning Process Analyser/solid-phase cytometry (Scan RDI™ in North America)
Chl *a*	Chlorophyll a
CICEET	Cooperative Institute for Coastal and Estuarine Technology
CPR	Continuous Plankton Recorder
CTD	Conductivity, Temperature, Depth
dH_2O	distilled water
DAPI	4’,6-diamidino-2-phenylindoline
DIC	Differential interference contrast
DICANN	Dinoflagellate Categorisation by Artificial Neural Network
DSP	Diarrhetic Shellfish Poisoning
ECOHAB	The Ecology and Oceanography of Harmful Algal Blooms
EPA	Environmental Protection Agency
ESD	Equivalent Spherical Diameter
ESP	Environmental Sample Processor
EU	European Union
EU DETAL	Rapid and ultra-sensitive fluorescent test for the tracking of toxic algae in the marine environment
FISH	Fluorescence *In Situ* Hybridisation
FIT	Fluid Imaging Technologies
FITC	Fluorescein isothiocyanate
FlowCAM	Flow Cytometer And Microscope
FSW	Filtered Sea Water
FTF	Filter-Transfer-Freeze
GF/F	Glass Fibre Filters
HAB(s)	Harmful Algal Bloom(s)
HAB Buoy	Harmful Algal Bloom Buoy
HAE(s)	Harmful Algal Event(s)
Hybe	Hybridisation
ICES	International Council for the Exploration of the Sea
IOC	Intergovernmental Oceanographic Comission of UNESCO
LED	Light-Emitting Diode
LM	Light Microscopy
LSU	Large SubUnit
MBARI	Monterey Bay Aquarium Research Institute
NASA	National Aeronautics and Space Administration
NOAA	National Oceanic and Atmospheric Administration
NSF	National Science Foundation
ONR	Office of Naval Research
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PICODIV	Picoplankton Diversity
PSP	Paralytic Shellfish Poisoning
QC	Quality Control
rDNA	Ribosomal Deoxyribonucleic Acid
RNases	Ribonucleases
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Real-Time PCR Please note that RT-PCR usually refers to reverse transcriptase PCR, In this manual the acronym refers to Real Time PCR.
SEM	Scanning electron Microscopy
SOP	Standard Operating Procedure
SSU	Small SubUnit
TEM	Transmission Electron Microscopy
TSA-FISH	Tyramide Signal Amplification has been used with FISH
UNESCO	United Nations Educational Scientific and Cultural Organisation
wt/vol	weight/volume

SI Unit	Meaning
I_2	Iodine
KI	Potassium Iodide
mL	millilitre
L	Litre
cm	Centimetre
hr	Hour
ºC	Degree Celsius
%	Percentage
µm	Micrometre
mm^2	Millimetre squared
Min^{-1}	Per minute
Hg	Mercury
n	Number
g	Gram(s)
µL	Microlitres
v/v	Volume to volume
x g	Multiplied by gravity

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