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George Dubelaar, Raffaella Casotti, Glenn A. Tarran, Isabelle C. Biegala

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Author queries for chapter 13 (Dubelaar et al)

■AQ1■ “Dignum 2001” is not listed in the reference section

■AQ2■ “Dignum et al 2004” has not been cited in the text

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13 Phytoplankton and their Analysis by Flow Cytometry

George B. J. Dubelaar, Raffaella Casotti, Glen A. Tarran,
and Isabelle C. Biegala

Overview

This chapter outlines how flow cytometry can be used for the analysis of phytoplankton: from basic and straightforward analysis to more challenging applications. Whereas most applications of flow cytometry in aquatic science are still laboratory-based, the emphasis of this chapter is on its potential to be used *in situ*, operated on a high frequency basis. This permits high-resolution sampling in time and space, which is crucial for our understanding of aquatic microbial ecosystems. Beginning with a basic description of the target particles, a sketch is made of the aquatic environment of these microorganisms and specific properties of the environment that may be very different from typical biomedical or plant research conditions. Some phytoplankton-related limitations and pitfalls are discussed as well as special instruments and instrument modification for phytoplankton analysis. Phytoplankton sampling is described with consideration to “critical scales”, including the use of platforms such as research ships and ships of opportunity, submerged use in vertical casts or on Autonomous Underwater Vehicles and moored platforms. Various applications are presented, including the screening of species, probing phytoplankton biodiversity, monitoring “harmful algal blooms”, studying population-related processes, and cell-related processes and functioning. Plankton abundance pattern in the sea is assessed and the coupling of flow cytometry data to ocean optics and physics is advocated. Finally, the potential use of flow cytometry in protection and warning is discussed, including the monitoring of harmful algal blooms, water quality in the aquaculture industry, bathing waters and the drinking water industry, as well as in the bio-indications and the control of ship’s ballast water treatment systems.

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13.1 Introduction

In as much as phytoplankton can be regarded as a “special type” of plant, this chapter should be regarded as a “special” addition to this book on plant flow cytometry. Its purpose is to introduce readers to the arena of aquatic flow cytometry, describing both the various applications and their biological significance as well as the unique features of flow instruments and methodology.

13.2 Plankton and their Importance

13.2.1 Particles in Surface Water

Surface waters in lakes, rivers, seas and the oceans contain a wealth of microscopic particles, both living and non-living. The non-living component consists of mineral particles and particles of biological origin such as dead cells, debris and organic matter. River water and water in coastal zones in particular, are often heavily loaded with mineral particles such as clay, silt and small sand grains. The living component is generally referred to as “plankton”: microscopic organisms, suspended in the water column. These living particles comprise viruses, bacteria, archaea and eukaryotic phytoplankton and zooplankton. Viruses are the most abundant biological agents in seawater. They infect bacteria, phytoplankton and zooplankton and may be important in controlling the abundance and composition of microbial communities. Bacteria are vital components of the aquatic microbial community. Many break down particulate matter, such as cells and detritus, and take up dissolved organic matter, converting it into cell mass, whereas some contain chlorophyll and other pigments and are photosynthetic. Despite being classified among the Bacteria, because of their photosynthetic activity cyanobacteria are considered to belong to the *phytoplankton*. The use of flow cytometry (FCM) for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities has been discussed by Gasol and Del Giorgio (2000).

Archaea are a newly emerging group of planktonic organisms (Giovannoni and Stingl 2005). They are prokaryotes, similar in size and shape to bacteria, being generally less than 1 μm in size. They are being found in many aquatic environments using molecular techniques, although their role in the plankton is still under investigation. Eukaryotic plankton can be divided into zooplankton and phytoplankton, with some species being mixotrophic. Many zooplankton are herbivores and graze on phytoplankton. Zooplankton are divided into protozoa and metazoa. The protozoa are made up of single celled flagellates, ciliates and sarcodines (amoebae and radiolarians), whereas the metazoans include rotifers, cladocera, copepoda, salps, and others.

13.2.2

Phytoplankton

The phytoplankton (algae s.l.) are planktonic plants occurring as single cells, colonies or filaments. Like all plants, they use carbon dioxide and light to produce sugar in the process of photosynthesis. Overall phytoplankton are responsible for 45% of primary production on earth and as such are considered to be a massive CO₂ pump owing to their photosynthetic activity. Phytoplankton together with the rainforests are the two “lungs” of our planet, playing a key role in gas exchange with the atmosphere. Central parts of the oceans, which are deprived in nutrients, are dominated by *Prochlorococcus*, a cyanobacterium about 0.8 μm in size, the tiniest phytoplankton and the most abundant photosynthetic organisms on earth. Due to their photosynthetic activity, the ancestors of this organism contributed to the origin of our oxygenated atmosphere. These discoveries, which contributed to the understanding of the functioning of our planet, could not have been possible without the help of FCM (Li 1995). On a smaller scale, the mineral enrichment of inland and coastal waters due to human activity leads not only to increased phytoplankton growth, but also to the occurrence of population explosions of nuisance species. The aquaculture industry is particularly vulnerable to phytoplankton damage.

Phytoplankton range in size from abundant sub-micron cyanobacteria to colonial and filamentous species that can reach several millimeters in length and are visible to the naked eye. Figure 13.1 gives an impression of their enormous size range. Phytoplankton are commonly subdivided into three size classes: picophytoplankton (<2 μm), nanophytoplankton (2–20 μm), and microphytoplankton (>20–200 μm).

The diversity of phytoplankton is very high and representatives of most algal divisions may be found among these aquatic organisms. The golden-brown algal line (containing chlorophylls *a* and *c*) includes diatoms and dinoflagellates, which belong to the best known and species-rich groups in the plankton. The green algal line (with chlorophylls *a* and *b*) includes green algae and euglenophytes, which are also very common in marine coastal and offshore waters. The most numerous phytoplankton, however, belong to the cyanobacteria (with chlorophyll *a* and biliproteins), which include free-living prochlorophytes (Urbach et al. 1992). These are ubiquitous and are able to thrive in very dim light at the base of the euphotic zone (Chisholm et al. 1988; Waterbury et al. 1979). The red algal line (with chlorophyll *a* and biliproteins) is common in benthic habitats of tropical reef waters and in Norwegian coastal waters (Paasche and Throndsen 1970), but is not generally detected in oceanic plankton.

13.2.3

Distributions in the Aquatic Environment

All the above-mentioned organisms as well as the non-living particles may occur in greatly varying abundances and compositions in the water column as compo-

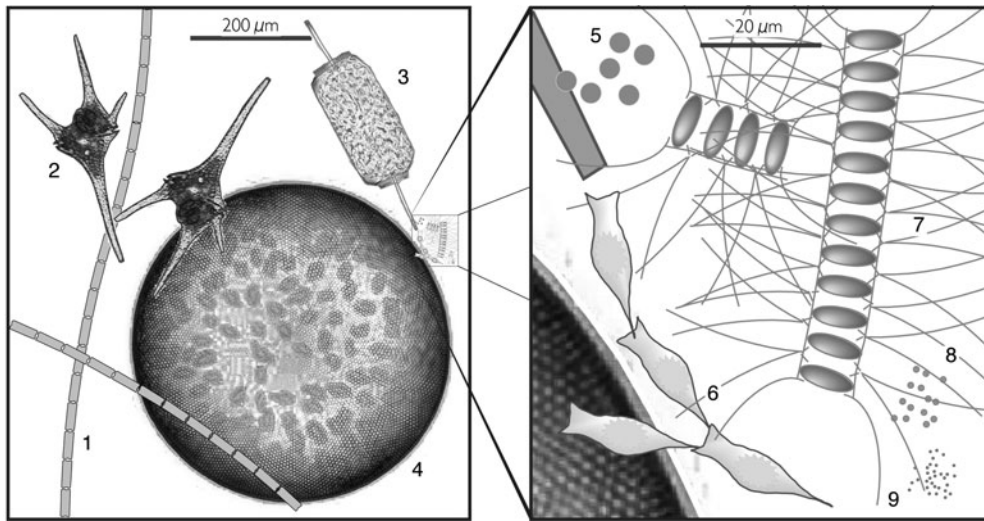


Fig. 13.1 Schematic drawings of some phytoplankton taxa illustrating their size differences. Bacillariophyta: (1) *Melosira*, (3) *Ditylum*, (4) *Coscinodiscus*, (7) *Chaetoceros*; Pyrrhophyta: (2) *Ceratium*; Chlorophyta: (5) *Chlorella*; Chrysophyta: (6) *Dinobryon*; Cyanobacteria: (8) *Synechococcus*, (9) *Prochlorococcus*.

nents of the microbial community, affecting other organisms by their presence and vice versa (predation) as well as being affected by physical and environmental factors and nutrients. Of crucial importance for the growth and succession of phytoplankton is the light penetration into the water column, which is in turn reduced by the living and non-living particles. Temperature is also an important condition with large diversity in the tolerance to variations in temperature among the algae, whereas turbulence plays a role as well. These factors lead to survival strategies such as colony formation, aggregation and buoyancy regulation. Throughout the annual cycle there is a temporal pattern in the development of communities of particular taxonomic composition and dominance hierarchy. Small microalgae are typically grazed by small invertebrates with comparable growth rates, rapidly responding to changes in prey density. Larger microalgae are grazed by copepods with growth rates an order of magnitude less, hence leading to temporally separated blooms. The composition of surface waters is intrinsically “patchy” and dynamic. Overall production by phytoplankton over an entire ocean can vary by 25% between years and up to a factor of 10 at a single location. Occurrence of single species may show much larger fluctuations. Patchiness is defined as variability in the range of 10 m to 100 km horizontally and/or 0.1 m to 50 m vertically in marine systems. Vertical variability such as “thin layers” (e.g. the “Deep Chlorophyll Maximum”, a temporally persistent and highly productive region near the bottom of the euphotic zone) are typical for stratified water columns. Storm cycles may in turn account for mixing, variance in water

movement and nutrient input. The persistence of “patchiness” is based on the interplay of patch-formation processes like population growth and patch-dissipation due to turbulence (Mackas et al. 1985).

13.3 Considerations for using Flow Cytometry

According to Peter Burkill from the National Oceanographic Centre, Southampton, UK, “Natural marine waters are complex with components that are often finely balanced. As man’s activities influence marine waters at an ever increasing rate, so it becomes increasingly important to understand their complexity and fine balance. Rapid, precise and objective techniques for the analysis of phytoplankton in marine waters are increasingly required. The stepping stone towards this lies with the development and deployment of suitable equipment. Flow cytometry clearly has much to offer here, although most instruments are suboptimal for the analysis of phytoplankton”.

13.3.1 Analytical Approach

The traditional method of analyzing phytoplankton populations with the use of microscopic techniques so that a trained scientist can recognize the morphology and size of different phytoplankton taxa (Utermohl 1958), is very time consuming and expensive. The sampling frequency and spatial coverage are generally low, resulting in limited data interpretation (Baretta et al. 1998; Smayda 1998). On the other hand, conventional quantitative oceanographic measurements only permit quantification of bulk properties of the water such as the concentration of chlorophyll *a* per m³. This provides at best only average properties of a hypothetical “typical” cell (Platt 1989). Our progress in understanding the functioning of microbial ecosystems and their response to external factors is being held back by these approaches. To analyze aquatic ecosystems in detail, it is crucial to collect information at the level of the single cell to account for biodiversity, viability, specific functions, and activities. This can be achieved using FCM due to its wide analysis range capability (from small zooplankton, phytoplankton and bacteria, to viruses) and its high count rate (thousands of cells per second).

Flow cytometer-based counting is accurate, direct and reliable, especially with instruments equipped with a volumetric sample delivery system (Rutten et al. 2005). When discrepancies occur with traditional methods, critical evaluation should follow. For example, validation trials were performed in the early 1990s in The Netherlands, comparing standard microscopic counting of the relatively easy recognizable *Rhodomonas* sp. (Cryptomonadaceae) cells in natural North Sea samples and FCM analysis of the same samples using the aquatic Optical Plankton Analyzer flow cytometer (Dubelaar et al. 1989). Large discrepancies between the FCM and microscope results were observed in the first year but they

largely disappeared in the second year, when transmission light microscopy (Utermohl 1958) was replaced by a fluorescence microscopy (Dubelaar et al. 2004).

13.3.2

Limitations and Pitfalls of using Biomedical Instruments

Aquatic scientists have achieved many great discoveries using FCM. However, this technology was designed and commercialized for biomedical applications. There are, therefore, a number of compromises that aquatic scientists had to make whilst using such instruments, as they were not designed with aquatic applications in mind. Some of these compromises are briefly mentioned below.

(i) Laser emission lines and filter set ups for fluorescence detection are not optimal for auto-fluorescent phytoplankton. Chlorophyll has a much larger Stoke's shift as compared to the fluorochromes normally used in FCM, with optimal excitation at 430–440 nm and emission at 680–690 nm.

(ii) Accurate cell counts and cell sizes are necessary for many ecological studies, but instruments are usually not very good at providing these (most instruments use a differential air pressure system for sample and sheath fluid delivery instead of a volumetric delivery system).

(iii) Particles in water span a very wide size range compared to mammalian cells commonly analyzed using FCM. Many instruments have sufficient sensitivity to detect the particles at the smaller end of the size spectrum (sub-micron) but only a few are able to cope with the particle sizes at the opposite end of the spectrum (1 mm and larger). Particle size-related problems are due to less efficient uptake from the sample flask into narrow sample tubing, maintaining them in suspension throughout the complete fluidics trajectory in the instrument, the risk of partial or complete clogging of an instrument as well as problems with sorting devices. Data deterioration and deviation from linearity is caused by incomplete illumination by the laser light and detector saturation with cells and colonies that are too large.

(iv) Particle concentrations encountered in aquatic samples also vary greatly. In eutrophic conditions phytoplankton blooms can reach densities of 10^6 cells ml^{-1} , which is similar to cell suspensions used in biomedical applications. High concentrations in the order of 10^7 particles ml^{-1} are typically reached by the smallest particles (i.e. viruses and sediment). In low nutrient oligotrophic waters, particle concentrations decrease, however, down to less than one individual per millilitre for the larger species. These low natural concentrations require high sample analysis rates, which are a problem at typical FCM sample rates of $0.2\text{--}2 \mu\text{l s}^{-1}$.

(v) Instruments are usually set up to measure discrete samples, while continuous sampling in a flow stream is useful for many aquatic applications.

(vi) The passage through a flow cytometer may damage vulnerable cells and colonies. High intensity laser light may cause photosynthetic shock and electrical shock may be caused by sorting. Large fragile particles may be broken by the high fluid shear in small nozzles.

(vii) The length of many phytoplankton species may also cause artifacts. For instance, the duration of signals of long cells and colonies present in phytoplankton samples is often truncated by the design properties of the instrument's electronics.

(viii) Another artifact can be due to non-homogeneously distributed chlorophyll. When a space between adjacent pigmented regions within a single particle (a large cell or a colony) exceeds the width of the laser focus, the fluorescence signal may drop below the triggering level, ending the processing of this particular particle prematurely. Large intercellular spaces within colonies occur in various species, for instance *Thalassiosira*, *Skeletonema* (both Bacillariophyta), and others; low pigmented heterocysts in filamentous cyanobacteria such as *Anabaena* may cause the same effect.

13.3.3

Instrument Modification and Specialized Cytometers

Various scientists have modified existing flow cytometers for use with phytoplankton samples, for instance to increase the sample flow rate and/or the dynamic range. Others have designed their own instruments. In 1983, Olson et al. (1983) described a flow cytometer for the analysis of fluorescence signals in phytoplankton consisting of an epifluorescence microscope, a photomultiplier, a "Coulter" size analyzer and an inexpensive quartz capillary flow system as the basic components. A low-cost portable flow cytometer with a wide (300 μm) nozzle was designed and built by Cunningham (1990a). Frankel et al. (1990) developed a high-sensitivity flow cytometer for studying picoplankton, suitable for shipboard FCM, and Hüller et al. (1991) reported on a macro flow planktometer for analysis of large marine plankton organisms (>100 μm). The analyses included electrical impedance volume, fluorescence and beam attenuation. A special instrument for measurements of field samples with a large particle size range (Optical Plankton Analyser) was reported by Dubelaar et al. (1989), Peeters et al. (1989), and Balfoort et al. (1992). A very wide range of particle sizes could be analyzed in one run, with a very low level of fluid shear, and a large sample flow.

Based on this instrument the "EurOPA" flow cytometer was developed (Dubelaar et al. 1995) with add-on features such as diffracted light pattern detection and analysis (Cunningham and Buonaccorsi 1992), pulse shape acquisition and analysis, imaging-in-flow (Wietzorrek et al. 1994), and more automated data analysis (Carr et al. 1994, Wilkins et al. 1996), particularly by artificial neural networks (Boddy 1994). Changing the approach to a compact and transportable design (Dubelaar and Gerritzen 2000; Dubelaar et al. 1999), the core technology was redesigned as a (commercially available) series based on a very small footprint instrument, available for various platforms such as a bench-top version (CytoSense) for static or mobile laboratories (ship) as well as a submersible version (CytoSub) for operation at depths of 200 m, and a moored (floating) version (CytoBuoy) placed inside a small spherical buoy with radio-transmission of data over line of sight distances (Fig. 13.2). The particle size range extends from submicron picoplank-



Fig. 13.2 Instruments designed for aquatic flow cytometry. (a) Submersible Flow Cytobot (H. Sosik and R. Olson, Woods Hole Oceanographic Institution, Woods Hole, MA, USA). (b) Submersible CytoSub (CytoBuoy b.v., Nieuwerbrug, Netherlands) mounted on

a rosette sampling frame. (c) Floating CytoBuoy (CytoBuoy b.v.), a flow cytometer inside a 90-cm spherical buoy for operation within line of sight (approximately 10 miles) from a land station. (d) Testing radio data transfer.

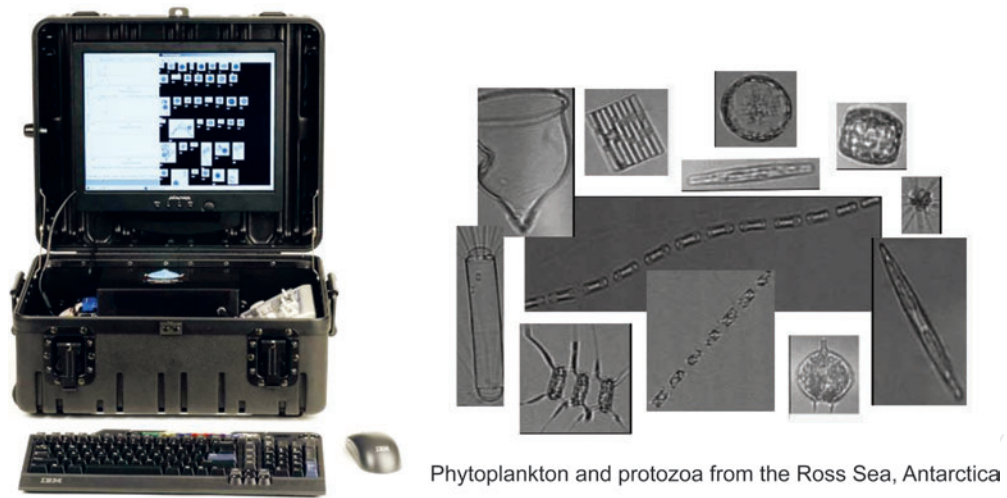
ton to the large diatoms and their chains and filaments. The data format includes the digitized pulses (one-dimensional scanned particle shapes) for morphological analysis of these particles. Another submersible instrument was developed by Olson et al. (2003). This automated submersible flow cytometer called Flow Cytobot (Fig. 13.2a) has been deployed at coastal cabled observatory sites for analyzing pico- and nanophytoplankton at relatively shallow depths. A hybrid instrument, FlowCAM of Fluid Imaging Inc. (Edgecomb, ME, USA) acquires images of (20 μm and larger) cells in a sample stream passing a fan of laser beams generating also light scatter and fluorescence signals (Sieracki et al.

1998). Different objectives can be used for different magnifications; the camera image is digitized and a sub-image containing the cell is stored in real time (Fig. 13.3a).

13.3.4 Sizing and Discrimination of Cells

Flow cytometers may provide several types of signal that can be used for sizing and characterization of measured particles. The most commonly used are forward light scatter, perpendicular (side) light scatter and fluorescence. Less often used are excitation light beam attenuation and the “time of flight”. The time of flight is the time a particle needs to cross the laser beam, which is proportional to the particle length (see also Chapter 2). The beam attenuation results from the removal of energy from the laser beam during passage of a particle. Although not a standard parameter, it can nonetheless be measured. Forward and side light scatter relate to size in different ways. The side scatter may yield the most straightforward relationship (proportional to particle cross-section) for particles $> 1 \mu\text{m}$ upward, with low refractive index (Morel 1991). However, it is known to be very sensitive to internal cellular structures. Forward light scatter is proportional to cellular cross-section only for very large cells ($>50 \mu\text{m}$), and highly absorbing cells, and shows variable behavior with decreasing size. The emitted fluorescence is proportional to the number of absorbed photons and the fluorescence quantum yield of a fluorophore. The absorption efficiency may vary from linearity with small particle size to a constant value for large particles; therefore calibration is required in order to use fluorescence as a size indicator.

The ratio between various fluorescence signals may be a useful discrimination tool. The presence of specific accessory antenna pigments varies between the main taxonomic phytoplankton groups. Fluorescence emission and excitation characteristics are influenced by these pigments and can be used to classify phytoplankton populations. Whereas high resolution spectrophotometric spectra refer to the bulk optical properties of a sample, FCM probes spectral properties of individual cells at high speed. Owing to the physical organization of the energy transfer between pigments, it is more efficient in terms of discriminatory power to analyze excitation spectra instead of emission spectra (Owens 1991). Unfortunately, there are seldom more than one or two laser wavelengths available to serve as an excitation source. On the emission side things are slightly better. The low duration and amplitude of the fluorescence signals of the individual particles allow only a crude spectral decomposition of the emitted fluorescence, and typically, three to five successive dichroic mirrors feeding different photomultiplier tubes (PMTs) are used. These excitation/emission data can be used to determine pigment ratio for each individual particle in a sample. The ratios are surprisingly small and stable for a healthy phytoplankton population and can be used to classify each individual into different taxonomic groups. Using three lasers as excitation light sources, Hofstraat et al. (1991) recognized cyanobacteria, cryptophytes, chlorophytes, and prasinophytes. Olson et al. (1989) used 488 and 515-nm excita-



Phytoplankton and protozoa from the Ross Sea, Antarctica

(a)

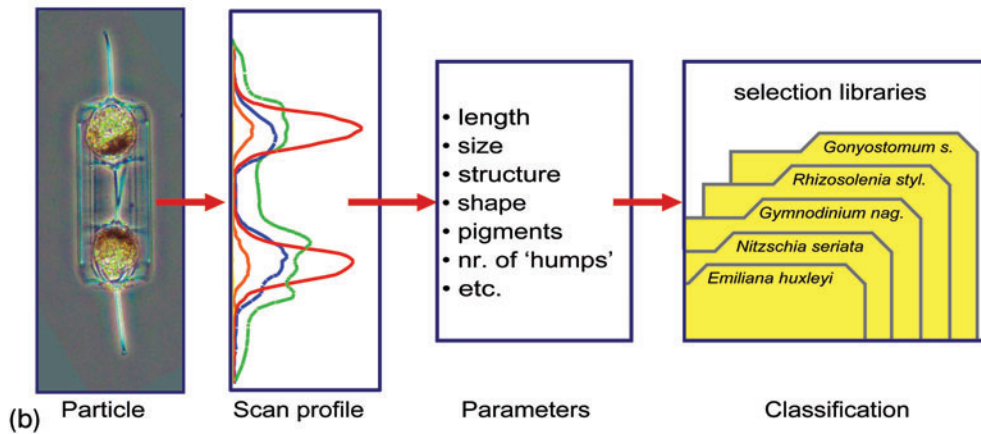


Fig. 13.3 (a) Example of data output of FlowCAM (from Kent Peterson, FlowCAM Technology: Digital Imaging Flow Cytometry for Oceanographic Research. Presentation at the ASLO/TOS Ocean Research 2004 Conference, February 2004, Honolulu, HI, USA; unpublished). (b) Principle of scanning flow cytometry. The sample is injected into a particle-free sheath fluid that narrows down the suspension into a very thin line of fluid in which the particles are gently stretched into a single file, moving at a fixed high

speed exactly through the middle of a focused laser beam. The amplitude, length and shape of the scatter and fluorescence signals represent the size, length and shape of the particles, as well as the distribution of its “body parts” and chloroplasts along its length axis. With chain-forming diatom colonies, the single cells show up as “humps” in the scan. After parameterization, this morphological information is analyzed by standard cluster analysis.

tion wavelengths and fluorescence detection at phycoerythrin and chlorophyll emission bands to distinguish cryptophytes, a rhodophyte, coccolithophorids, and chlorophytes in a mixture of 26 cultures. Similarly, a high side scatter to forward scatter ratio can be used to discriminate cells with high refractive index, such as the calcite coccoliths surrounding coccolithophores or the intracellular gas vacuoles in certain species.

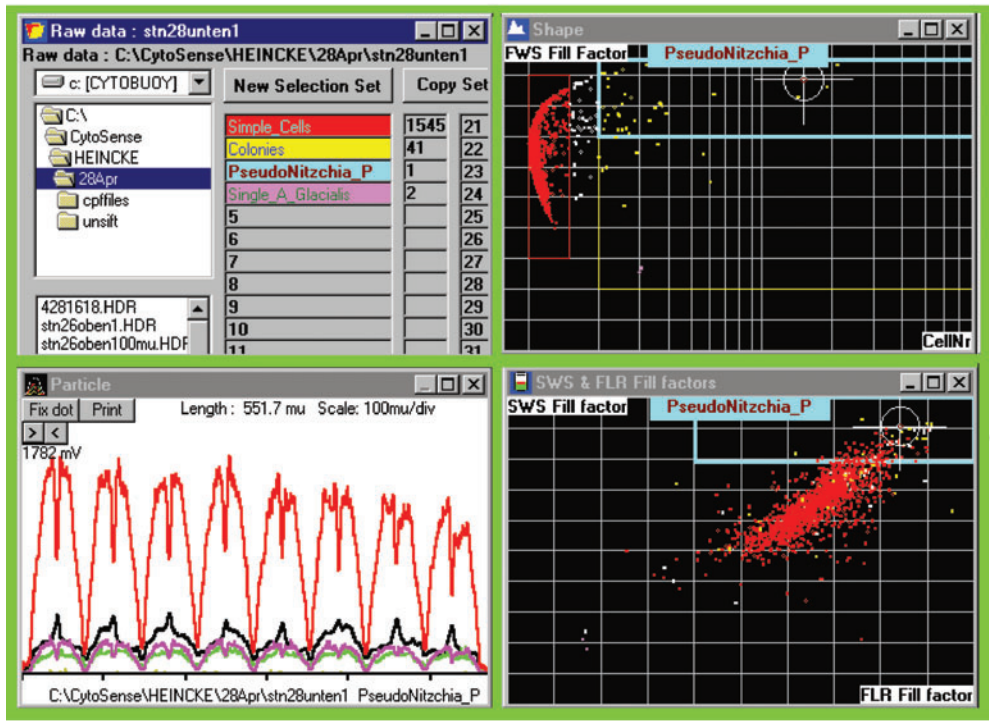
13.3.5

More Information per Particle: From Single Properties to (Silico-) Imaging

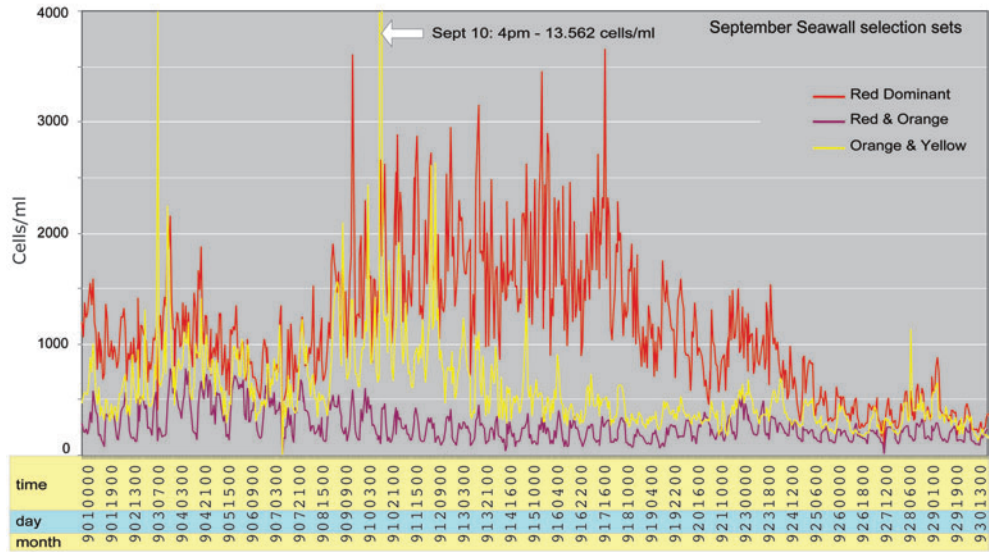
Data analysis is a bottle neck in many applications involving FCM in aquatic sciences. In the biomedical field, the cytometrist is usually faced with only a few cell types to differentiate, whereas in aquatic situations the number of cell types is typically up to an order of magnitude greater. In addition, all these species appear in greatly varying concentrations, in different life cycle stages, in different states of aggregation and are typically accompanied by large numbers of debris and non-biological particles. With regard to assessing the microbial composition, it is necessary to count and identify as many groups and species as possible. As diversity increases, the number of measured particles per sample has to increase accordingly, to allow for clustering (requirement for subpopulation detection and analysis) of the less abundant species. This implies the generation of sets of measured data for tens of thousands of particles per sample. At the moment, autofluorescence at various excitation/emission wavelengths, together with measured light scattering properties are used to differentiate between species, keeping the data set per measured cell limited to only three to six numbers. With more detectors the amount of data per particle increases and combined with high numbers of particles to be analyzed, data overload may become a problem. One method to cope with this is artificial neural networks (ANNs). Boddy et al. (2000) presented an ANN-based analysis of 72 cultured species in a mixed sample with an overall identification rate of at least 70% using standard FCM data based on seven optical parameters. Wilkins et al. (1999) have shown a 92% success rate for 34 somewhat more distinct species measured with an 11-parameter flow cytometer.

FCM data provides less complex information compared to microscopy. However, cytometry can offer more detailed analysis of phytoplankton by deploying morphological and/or physiological analysis techniques such as forward light diffraction analysis, photosystem probing, pulse shape analysis (can be regarded as one-dimensional scanning or “silico-imaging”), and traditional imaging. The discriminatory potential depends on the number of independent properties measured for each particle, as well as the parameterization of these properties. The latter is also crucial for the overall applicability of the technology in terms of automated data analysis and the processing of large data sets.

An interesting development has been the “Pump-During-Probe” flow cytometer (Olson et al. 1996) in which the time course of chlorophyll fluorescence yield is measured during a 150-ms excitation flash provided by an argon ion laser. This provides estimates of the potential quantum yield of photochemistry and the



(a)



(b)

functional absorption cross-section for photosystem II, for either individuals (in large cells) or populations (in small cells).

Another approach, which is similar in data load but more straightforward in terms of instrumentation, is one-dimensional scanning FCM (also called “silico-imaging”). In FCM, signals coming from light detectors are normally quantified only by their maximum height and area. However, it is relatively easy to digitize and store the complete signal profiles in real time for morphological analysis (Fig. 13.3b). The information thus obtained relates to the shape of the particles in a format that allows efficient processing of large data sets (Cunningham 1990b; Dubelaar et al. 2000; Rutten et al. 2005). For example, the data cluster analysis application CytoClus operating with the five-parameter flow scanning cytometer CytoSense (CytoBuoy b.v., Netherlands), reduces each of the five digitized pulse profiles per particle to six parameters: the pulse length and average amplitude, the fill factor, the asymmetry, the inertia, and the number of “humps” in the profile, which is roughly the number of cells in a filamentous/chain forming colony. From these 30 (five profiles \times six parameters) available parameterized values for each particle, only 14 have been used so far in the data processing software, allowing typically between 30 and 50 groups of particles in natural samples of fresh and marine waters to be distinguished.

The discrimination power is shown with data from a cruise on the North Sea, on the R/V Heincke (Alfred Wegener Institute (AWI), Germany) in April/May 2003. Samples were collected at various depths at successive stations. During night time, samples were analyzed at 10-min intervals from a sea water supply hose. Based upon selection criteria obtained from a data file of a culture of *Pseudo-nitzschia pungens* (Bacillariophyta), a set of measured data files were screened for the presence of *P. pungens*. This species was occasionally found in low numbers. An example of a single “matching result” is shown in Fig. 13.4a. Table 13.1 shows how the pulse profile information increases the discrimination power for the detection of *Pseudo-nitzschia* in 10 samples. Adding the length to the “classical” parameters (not shown separately) resulted in a reduced number of false positives, but only after screening with the full pulse shape parameter

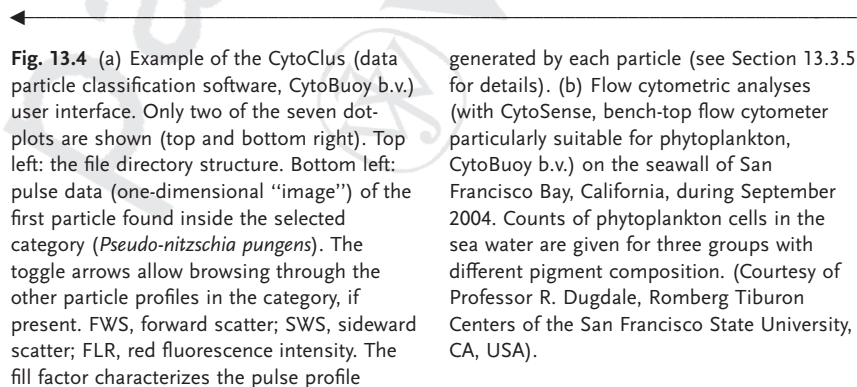


Fig. 13.4 (a) Example of the CytoClus (data particle classification software, CytoBuoy b.v.) user interface. Only two of the seven dot-plots are shown (top and bottom right). Top left: the file directory structure. Bottom left: pulse data (one-dimensional “image”) of the first particle found inside the selected category (*Pseudo-nitzschia pungens*). The toggle arrows allow browsing through the other particle profiles in the category, if present. FWS, forward scatter; SWS, sideward scatter; FLR, red fluorescence intensity. The fill factor characterizes the pulse profile generated by each particle (see Section 13.3.5 for details). (b) Flow cytometric analyses (with CytoSense, bench-top flow cytometer particularly suitable for phytoplankton, CytoBuoy b.v.) on the seawall of San Francisco Bay, California, during September 2004. Counts of phytoplankton cells in the sea water are given for three groups with different pigment composition. (Courtesy of Professor R. Dugdale, Romberg Tiburon Centers of the San Francisco State University, CA, USA).

Table 13.1 CytoSense (CytoBuoy b.v., Nieuwerbrug, Netherlands) data from 10 stations on a North Sea cruise (on the Heincke, AWI, Alfred Wegener Institut, Bremerhaven, Germany, April/May 2003). *Pseudo-nitzschia* colonies were identified from the total number of measured fluorescent particles by using a selection set based on classical flow cytometry parameters first (pulse height and area), followed by adding the pulse length parameter only and then by adding more pulse shape parameters (see Section 13.3.5 for details).

| Heincke cruise | Number of individuals | | |
|-------------------|-----------------------|--------------------------------|---------------------------|
| | Fluorescent particles | <i>Pseudo-nitzschia</i> set | |
| File name | | Classic properties plus length | Same set with pulse shape |
| Station 26 | 3396 | 74 | 1 |
| Station 27 top | 3877 | 28 | 0 |
| Station 27 bottom | 3187 | 120 | 1 |
| Station 28 top | 3243 | 53 | 0 |
| Station 28 bottom | 3148 | 41 | 1 |
| Station 29 top | 5321 | 83 | 4 |
| Station 29 bottom | 4480 | 60 | 2 |
| Station 30 top | 5181 | 29 | 0 |
| Station 30 bottom | 3935 | 30 | 0 |
| Station 31 bottom | 6766 | 8 | 0 |
| Totals | 42534 | 526 | 9 |

set did the number of positively identified *Pseudo-nitzschia* colonies match with reality. “Silico-imaging” (in contrast to video-imaging) runs at full flow cytometer particle throughput rate (up to 1000 or more particles per second) and full particle size range (submicron to millimetre), which allows accurate determination of particle assemblage composition and concentrations at high throughput rates.

To acquire video images of cells and store these alongside the more usual light scattering and fluorescence measurements is very helpful for identification of measured particles: clusters in data space are revealed. This was first tested with the EurOPA instrument (Dubelaar et al. 1995), which featured “imaging-in-flow” of pre-selected cells for identification of clusters found in data space. This allowed relatively fast identification of clusters, but it remains an interactive procedure in which the user examines a series of images (Rutten et al. 2005). A similar development is the FlowCAM (Fluid Imaging Inc.), which acquires video images of (20 µm and larger) cells in a sample stream passing a fan of laser light yielding light scatter and/or fluorescence signals serving as trigger source (Sieracki et al. 1998). The software allows grouping of the acquired images on the basis of basic geometrical properties with cell sizes being measured directly from the images.

13.4 Sampling: How, Where and When

13.4.1 Sample Preparation

The size of a sample and the number of steps involved in sub-sampling affect the significance of the results in a statistical manner. Whereas the typical FCM sample volume is only tens to hundreds of microlitres, the analyzed number of particles can be up to hundreds of thousands. The design of a sampling strategy therefore depends on the type of environment the samples are taken from and the relative concentration of the target particles. FCM samples in the biomedical laboratory typically have concentrations of about 10^6 particles ml^{-1} . These concentrations can often be attained by bacteria, particularly in freshwater systems and coastal waters, and are the norm for viruses in aquatic systems. On the other hand, phytoplankton only reach such high numbers in exceptional cases such as dense blooms. Most natural phytoplankton samples have concentrations of cells lower by two to three orders of magnitude. Small cells typically occur at higher concentrations, whereas the larger species may bloom at cell densities of only a few thousands per litre. One way to increase the number of cells counted is to enrich samples before the analysis. However, enrichment of samples by filtration or centrifugation inevitably leads to a change in the relative frequency of particles. For example, Hofstraat et al. (1990) examined the effect of simple filtration, tangential filtration and centrifugation on the length of *Skeletonema costatum* colonies. It was shown that damage to fragile particles, especially the largest colonies, was unavoidable. Centrifugation caused the least damage but was still not perfect.

In many aquatic applications, the analysis is carried out in the laboratory. Live samples quickly degrade, altering community composition, so it is generally necessary to preserve samples and transport them from the study site back to the laboratory. Many methods for the preservation of phytoplankton samples have been developed in the past, but none have been ideal or generally applicable. Formaldehyde and Lugol's iodine fixation modify cell shape and drastically affect fluorescence, respectively; ethanol fixation results in photosynthetic pigment extraction and therefore loss of cell autofluorescence. Low concentrations of glutaraldehyde and formaldehyde perform well for analysis of freshwater samples within about 7 days. However, this is not sufficient for many marine applications as research cruises often last for many weeks. Vaultot et al. (1989) described a method consisting of 1% (v/v) glutaraldehyde fixation followed by storage of the samples in liquid nitrogen. This method works well with picoplanktonic populations. However, a significant proportion of larger and more fragile cells are usually lost. A protocol, consisting of immediate fixation with 0.1 to 0.5% (w/v) formaldehyde and storage at 4 °C, was reported by Premazzi (1992) and tested using cultures of the dinoflagellate *Gymnodinium corii*. Size and chlorophyll autofluorescence of the cultures were preserved very well for up to 4 months. This study concerned only a single species and is not representative of heterogeneous phytoplankton

communities. Recently, a very simple protocol involving a surfactant Pluronic F-68 has been developed, which allows picoplankton sample enrichment for FCM. This protocol can be used for the least represented group of picoeukaryotes (down to 10^2 cell ml^{-1}) avoiding any cell loss or cell damage (Biegala et al. 2003). Generally, the ideal situation is to analyze fresh samples soon after collection without pre-concentration. Where this is not possible, the data obtained from stored samples should be interpreted with caution.

13.4.2

Critical Scales and Sampling Frequency

“Critical scales” are the temporal and/or spatial scales at which data must be collected in order to resolve patterns and processes. If the sampling is not done at critical scales, the fundamental patterns of distribution of organisms in the water environment remain obscure as well as the processes which control the distributions and the dynamics over a broad range of temporal and spatial scales (Donaghay 2004). Taking bottle samples for plankton counting and taxonomy can be considered as taking a snapshot in time/space of the continually fluctuating state of the ecosystem. It is tempting to interpret the resulting data point as if it represents a realistic (average) value for that time period/area. The reliability of such an approach is inversely proportional to the “under-sampling” of the actual ecosystem variability. Therefore, ideally the relevant ecosystem variability (the critical temporal scale) should be known before deciding on a sampling regime.

In aquatic ecosystems, water movement and tidal currents may generate significant fluctuations with time constants of minutes to hours. It is well known that small microbes may double their numbers in a few hours. Consequently, in both stable and/or well-mixed situations significant changes may appear within one to a few days, depending on environmental factors such as sunlight, temperature and ecological factors such as grazing and viral attack. Sampling frequency, therefore, is an important consideration when undertaking any form of temporal or spatial study. For example, daily FCM analysis of over 30 phytoplankton groups and taxa in surface water (Oude Rijn canal, The Netherlands) showed that certain species can bloom and begin to disappear again within a week, whereas others remained constant (Dubelaar et al. 2004). Higher frequency sampling can reveal even finer scale changes in plankton communities. Hourly FCM analysis of phytoplankton abundance of some major groups of phytoplankton was carried out from the seawall at the mouth of the San Francisco Bay during September 2004 (R. Dugdale et al., personal communication). Sampling was achieved by pumping seawater samples up to the flow cytometer. The data show strong hourly fluctuations governed by the tidal movement (Fig. 13.4b).

In water quality monitoring applications, conducted by government agencies and other regulatory bodies, sampling frequencies rarely exceed two per month. Time series of phytoplankton counts at higher sampling frequency are scarce. Li and Dickie (2001) reported a multi-year series of phytoplankton counts of the Bedford Basin near Dartmouth, Nova Scotia, with a weekly sampling frequency. This series shows considerable ecosystem fluctuations, which would have been

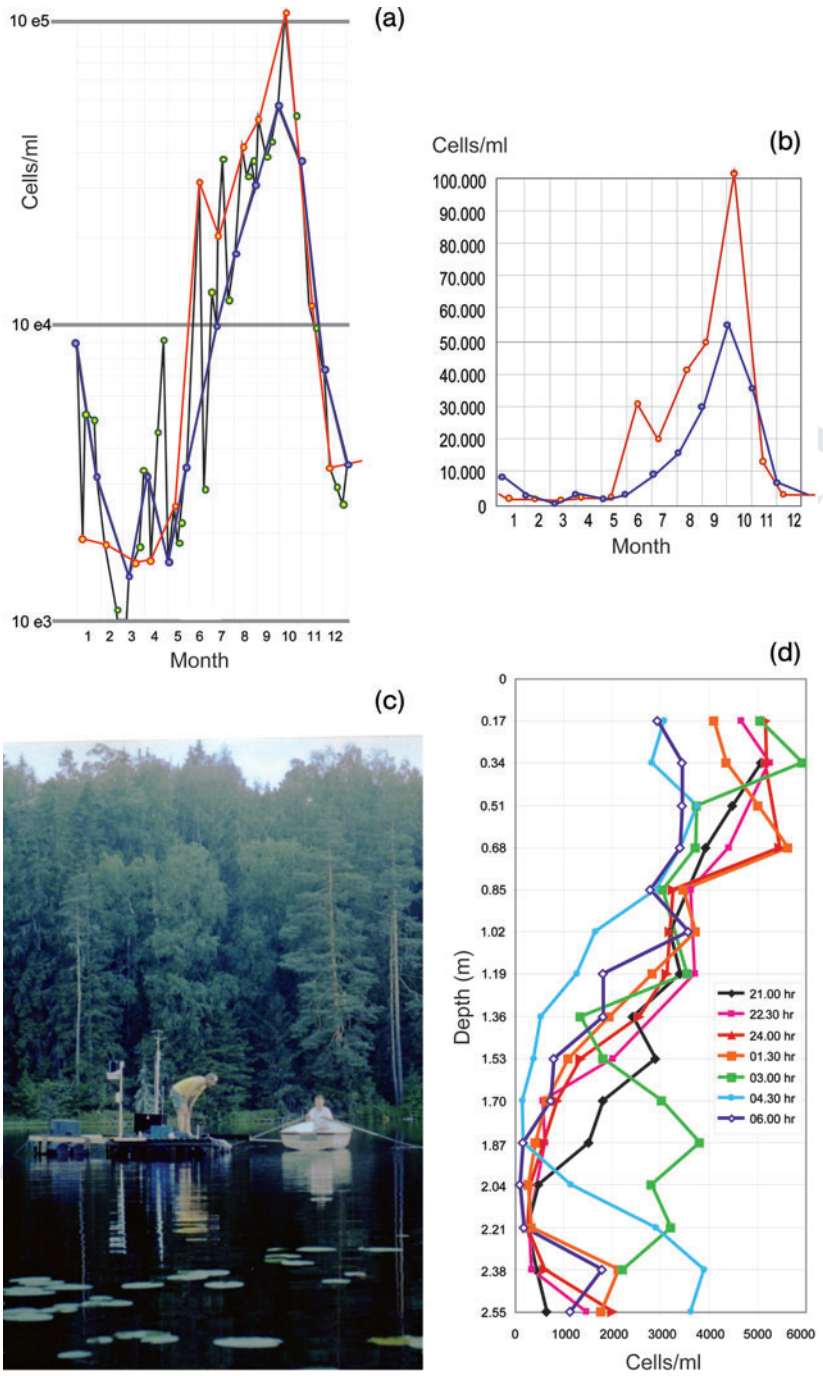
missed almost entirely if the sampling frequency had been reduced to once or twice a month. Figure 13.5a shows the number of phytoplankton cells in the Bedford basin during 2000 (Li and Dickie 2001). Monthly sampling was simulated by phytoplankton analysis every first and every second week of the month respectively. The aliasing caused by under-sampling results in two significantly differing graphs (Fig. 13.5b) both representing the same data. On many occasions the critical scales are not met as the sampling frequency is a compromise between a need to obtain sufficient resolution to understand what is going on, and the feasibility and costs involved to conduct the study. FCM may play an important role in finding the critical scales and to aid in designing adequate sampling and measurement programs for research and monitoring of surface waters with regard to analysis of microbial assemblages.

13.4.3

Platforms for Aquatic Flow Cytometry

Potential applications of FCM are to analyze phytoplankton over wide spatial scales on moving platforms such as ferries and research vessels. There are two approaches for operation onboard ships: rugged bench-top instruments for operation onboard and submersible instruments for operation on the ship's winch. The bench-top instruments can be used interactively measuring discrete samples taken at stations at various depths using a rosette water bottle sampler. Online autonomous sampling is the method of choice if a continuous sea water supply is available and the ship is on passage, effectively conducting transects, such as is usual with ferries and other ships of opportunity. A submersible flow cytometer can be lowered down the water column for real *in situ* analyses, for instance directly probing the deep chlorophyll maximum found at the bottom of the mixed layer in the open ocean. These "Thin Layers" – structures of the water column – were discovered by sampling coastal marine waters at "critical scales" and may only be a few centimeters to a meter in vertical depth, but in the order of kilometers in the horizontal extent. They have important implications for marine ecology, and for ocean optics and acoustics. In comparison to ship-operated profilers, bottom-up profilers are better suited for precision measurements (Donaghay 2004), such as those needed to locate layers of high abundance of certain species that may be as thin as 10 cm while spatially coherent over hundreds of meters. The bottom-up profilers consist of an anchored bottom station from which a slightly buoyant instrument package is reeled upwards while taking (semi-)continuous measurements.

Autonomous Underwater Vehicles (AUVs) are an emerging platform for analytical instruments to observe the marine environment over wide spatial scales. This will be of great value to researchers interested in fields such as oceanography, macro-ecology, and marine ecosystems. An example is AutoSub, a UK Government-funded AUV capable of collecting physical, biological, chemical, and geophysical data to depths of 6000 m and over transects of up to 8000 km (see insert in Fig. 13.8b). A CytoSub submersible flow cytometer was tested in the AutoSub, cruising through a coccolithophore bloom SE of the Isles of Scilly



(white “cloud” in the satellite image). The coccolithophore cells of *Emiliana huxleyi*, although outnumbered by other smaller species dominate the water leaving back scattered light as seen by the satellite, owing to their strongly scattering intracellular calcite plates (liths). Because the various groups/species can be distinguished from each other by FCM, this enables the determination of not only the cell numbers but also the fluorescence (pigment) and light scattering properties per group. The data from the flow cytometer onboard AutoSub confirmed that the coccolithophore cells were mostly causing the light reflection. The coccolithophore cell counts corresponded well with the turbidity and backscatter measurements (Cunningham et al. 2003).

Another mode in which specialized flow cytometers may be used is as a real-time monitoring tool in a fixed *in situ* location. Special flow cytometers, configured for autonomous operation, may be placed on floating moored platforms provided there is electrical power, data transmission capability and instrumental options. Data transmission, whilst feasible, is very expensive due to the high data load. However, most applications are near-shore. This means large data sets can be easily transferred by line of sight radio communication. Besides the CytoBuoy (Fig. 13.2c), small scale experiments may also be carried out with a waterproof or even a splash-proof bench-top instrument mounted on a floating platform. An experiment was performed with a CytoSense instrument on a small raft in a shallow stratified Finnish lake (Fig. 13.5c, d). A series of depth profiles of 15 depths at 17-cm intervals was taken automatically using a hose system lowered with a fishing rod reel with the FCM measuring in an on-line mode. Each 1.5 h a complete cycle was performed over all depths. In this way it was possible to study the fine-scale phytoplankton composition as a function of the water depth at high frequency.

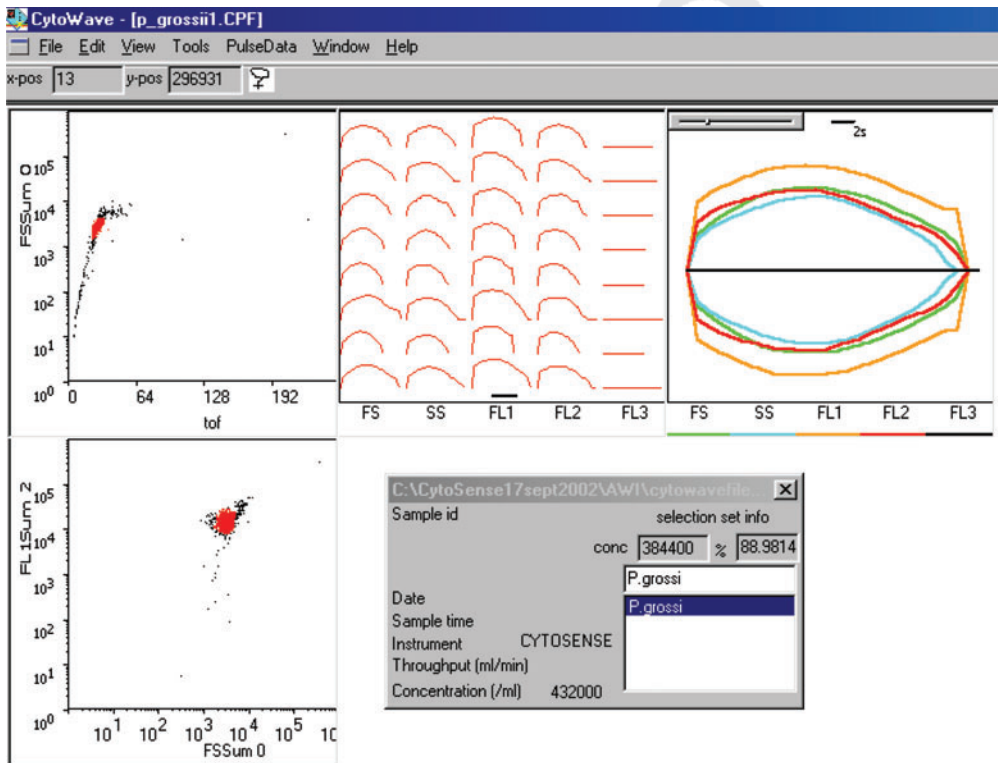
13.5 Monitoring Applications

13.5.1 Species Screening: Cultures

Many institutes and university departments in the field of marine, coastal and limnological research maintain culture collections of phytoplankton species and

Fig. 13.5 Weekly phytoplankton cell counts obtained using flow cytometry in the Bedford Basin, Canada, Atlantic Coast, from January to December 2001 (based on data of Li, 2001). (a) Black line represents original data, the red and blue lines represent sampling every first and second week of the month, respectively. (b) The resulting graphs on a linear scale. (c) CytoSense (CytoBuoy b.v.) being transported to the platform, Lake Lammi, Finland, July 2002, and (d) Series of successive depth profiles for one of the ca. 20 groups of particles/organisms that were identifiable in this particular data set.

strains. The conditions of the cultures are often not well known. FCM is a powerful diagnostic tool for rapid screening of the status of these cultures. Information on cell concentration, cellular properties and their statistics such as the mean and variance of fluorescence (pigment status) and light scatter (size, shape, physiology) can be directly obtained. Contamination with cells of a different species or bacteria can easily be detected and quantified. Figure 13.6 shows the result of an analysis of a sample containing *Pyramimonas grossii* (Chlorophyta). Various scat-



(a)

| SelectionSet name: | Concentration | | Forward scatter | | Signal (particle) length | | Fluorescence | |
|--------------------|--------------------|----------------------|-------------------|--------------|--------------------------|--------------|-------------------|--------------|
| | all particles #/ml | selection set % #/ml | mean value (a.u.) | Coef. Var. % | mean value μm | Coef. Var. % | mean value (a.u.) | Coef. Var. % |
| P. grossii | 432000 | 89 384400 | 3018 | 18 | 7 | 9 | 16030 | 26 |

(b)

Fig. 13.6 Flow cytometry data from a culture of *Pyramimonas grossii*. (a) This example (software: CytoWave, CytoBuoy b.v.) shows two scatter plots in which the *P. grossii* cluster is highlighted in red. The insert shows the concentration. The pulses overview (top middle) shows that the culture is very uniform, containing only single cells. In the pulse detail view (top right) it is possible to scroll through pulses from individual cells. (b) Statistical output for the data.

ter plots can be chosen using available measured entities such as scatter or fluorescence. The corresponding statistics can be directly presented using a standard spreadsheet program.

Phytoplankton species forming large colonies pose problems to conventional microscopic and automatic particle analysis techniques and most flow cytometers also have strict upper particle size limits that are too small for many phytoplankton colony formers. Instruments for large size ranges that collect images or scans of particles may be used to study the population dynamics of colony formers, filamentous algae and chain-forming diatoms. The length of particle pulse profiles as measured with a scanning cytometer is directly related to the particle's length. The number of cells in a chain can be obtained by Fourier analysis of the pulse profiles. Scanning flow cytometers are also useful for the analysis of species forming non-linear colonies of more or less identical cells. The size of fluorescence distributions can be normalized to that of a mean single cell even for more or less amorphous aggregations such as cyanobacterium *Microcystis aeruginosa* (Dubelaar et al. 1995). The kinetics of (dis)aggregation processes can also be monitored, even rapid changes, if the sampling frequency is sufficient.

13.5.2

Phytoplankton Species Biodiversity

Screening for specific target species in natural waters can be done well with FCM if these species are distinct enough to distinguish them from the non-target counterparts and their concentration is sufficient to have a significant count after a reasonable time of sampling. Although most commercially available flow cytometers are not suited to measuring large volumes of sample fluid, some instruments were designed for a high flow rate. With appropriate automation and real-time data reduction, such instruments may operate over longer periods of time without operator intervention to process larger sample volumes while looking for "target" species, either in the laboratory or *in situ*. What remains is the uniqueness of the target particle as measured by the flow cytometer.

Currently the most promising instrument to assess both aspects of biodiversity, i.e. species richness and abundance of species seems to employ molecular methods (Biegala 2003; Collier 2000; Not et al. 2004; Romari and Vaulot 2004; Simon et al. 1994; Vaulot et al. 2004). The most direct approach is to develop a species-specific agent to which a fluorescent label can be attached. Thus, Vrieling and Anderson (1996) and Vrieling et al. (1995, 1996, 1997) showed, that antisera against purified cell walls and against extruded trichocystal cores of the organism allowed immunofluorescent detection of the dinoflagellates *Prorocentrum micans*, *Gyrodinium aureolum* and *Gymnodinium nagasakiense*. An even more promising strategy involves the use specific oligonucleotides (Jonker et al. 2000; Simon et al. 1997). These can be used as primers for quantitative PCR (polymerase chain reaction), dot blot hybridization, and whole cell (i.e. *in situ*) hybridization. Each technique possesses its advantages and drawbacks, but among them fluorescence *in situ* hybridization of oligonucleotide probes (FISH) is the most straightforward and easiest to use. Once fluorescently tagged, cells can be enumerated by different methods.

Epifluorescence microscopy is the most commonly used method, confocal microscopy allows detection of cells associated with particles, and FCM allows a rapid enumeration of a large quantity of cells. The sorting capacities of new flow cytometers have led to the acquisition of more phylogenetic information on a fluorescently-tagged population to assess their detailed species richness.

13.5.3

Harmful Algal Blooms (HABs)

Blooms of toxic algae have been commonly called “red tides”, due to the fact that blooms are often caused by dinoflagellates whose pigments tint the water with a reddish color. The scientific community now uses the term “harmful algal bloom” or HAB. HABs are caused by a diverse group of organisms with serious impacts for humans and coastal ecosystems, including the dinoflagellates *Alexandrium tamarense* (paralytic shellfish poisoning), *Dinophysis* (diarrhetic shellfish poisoning), *Pfiesteria piscicida* (kills fish at mid-Atlantic latitudes), *Karinia brevis* (= *Gymnodinium brevis*; neurotoxic shellfish poisoning), diatoms such as *Pseudo-nitzschia* sp. (amnesiac shellfish poisoning) and *Chaetoceros* sp. (kills fish), pelagophytes such as *Aureococcus anophagefferens* and *Aureoumbra lagunensis* (brown tides), and cyanobacteria such as *Anabaena*, *Aphanizomenon*, *Microcystis*, and *Synechococcus elongatus* (harmful cyanobacterial blooms). Prevention of HABs is unlikely, although “control” seems feasible in the future. Mitigation as an effective strategy is well established. A rapid and reliable method for the specific detection of harmful algal strains is still badly needed. Molecular probes to external and internal cell features (as previously described in Section 13.5.2) are one way forward. Currently, probes can be used in conjunction with FCM in laboratory-based operations. Whereas standard instruments can be used as interactive discrete sampling devices, some “aquatic” cytometers can be deployed as continuous, automated *in situ* systems. These instruments discriminate the target species on the basis of their morphology, which may be specific for certain species (such as *Pseudo-nitzschia*) but less specific for others. A CytoSense analysis of a series of cultures from the collection of the AWI showed that the recognition of several types of Dinophyceae was very specific and almost no false positives were generated by the other cultures (Table 13.2).

13.6

Ecological Applications

13.6.1

Population-related Processes

Whilst monitoring and surveying programs involving quantification and classification of phytoplankton is generally targeted at specific species of interest, fundamental research in aquatic systems also employs flow cytometers to classify and

Table 13.2 The results of analysis of some cultures from the AWI Bremerhaven measured with a CytoSense flow cytometer. Narrow selection sets were determined for *Alexandrium minutum*, *A. catanella* and *Gymnodinium nagasakiense*. The vertical columns show how many individuals from other cultures match those in the selection sets (false positives).

| Cultures from AWI | | Sample volume (ml) | Total number of particles | Number of particles found matching the selection criteria | | |
|---------------------------------|----------|--------------------|---------------------------|---|------------------------------|--|
| Taxon designation | Code | | | <i>Alexandrium minutum</i> AL1T | <i>A. catanella</i> BAHME255 | <i>Gymnodinium nagasakiense</i> PLY561 |
| • | | | | | | |
| <i>Synura uvella</i> | CCMP870 | 0.015 | 1393 | 0 | 0 | 4 |
| • | | | | | | |
| <i>Cyclotella caspia</i> | | 0.014 | 1042 | 0 | 0 | 0 |
| <i>Cyclotella cryptica</i> | | 0.013 | 1316 | 0 | 0 | 0 |
| <i>Cyclotella meneghiniana</i> | | 0.016 | 1234 | 0 | 0 | 2 |
| <i>Cyclotella</i> sp. | 1435 | 0.024 | 1585 | 0 | 0 | 0 |
| <i>Alexandrium catenella</i> | BAHME255 | 0.276 | 675 | 9 | 97 | 0 |
| <i>Alexandrium minutum</i> | AL1T | 0.095 | 607 | 325 | 0 | 0 |
| <i>Alexandrium ostenfeldii</i> | LF37 | 0.89 | 475 | 0 | 1 | 0 |
| <i>Alexandrium tamarense</i> | GTPP01 | 0.18 | 938 | 3 | 0 | 7 |
| <i>Alexandrium tamarense</i> | SNZB01 | 0.644 | 667 | 19 | 19 | 0 |
| <i>Alexandrium taylori</i> | AY2T | 0.854 | 1061 | 0 | 0 | 0 |
| <i>Gymnodinium fuscum</i> | CCMP1677 | 0.463 | 338 | 0 | 0 | 0 |
| <i>Gymnodinium nagasakiense</i> | PLY561 | 0.131 | 545 | 0 | 0 | 315 |

Table 13.2 (continued)

| Cultures from AWI | | Sample volume (ml) | Total number of particles | Number of particles found matching the selection criteria | | |
|---------------------------------|--------------|--------------------|---------------------------|---|------------------------------|--|
| Taxon designation | Code | | | <i>Alexandrium minutum</i> AL1T | <i>A. catanella</i> BAHME255 | <i>Gymnodinium nagasakiense</i> PLY561 |
| <i>Gymnodinium nagasakiense</i> | GymNagas0204 | 0.051 | 695 | 0 | 0 | 219 |
| <i>Gymnodinium nagasakiense</i> | GymNagas0403 | 0.249 | 834 | 0 | 0 | 86 |
| <i>Gymnodinium nagasakiense</i> | GymNagas0204 | 0.085 | 667 | 1 | 0 | 246 |
| <i>Gymnodinium varians</i> | CCMP421 | 0.165 | 1393 | 0 | 0 | 0 |
| <i>Gyrodinium aureolum</i> | K0303 | 0.436 | 891 | 5 | 0 | 2 |
| <i>Heterocapsa triquetra</i> | CCMP448 | 0.097 | 1093 | 0 | 0 | 0 |
| • | | | | | | |
| <i>Synedra</i> sp. | | 0.01 | 1222 | 0 | 0 | 1 |

quantify phytoplankton, as well as bacteria and, more recently, viruses. This basic abundance data are used to quantify the standing stocks of phytoplankton in aquatic ecosystems from lakes and rivers to ocean basins (Tarran et al., 2006). FCM can also be used to study bloom development and succession by monitoring changes in phytoplankton standing stocks (Burkill et al. 2002; Tarran et al. 2001), and also to study controlling factors in ecosystem function, such as grazing and virus attack.

For example, phytoplankton can, through their autofluorescent characteristics, be thought of as tracer particles in much the same way as fluorescent microspheres when used in particle uptake experiments. Phytoplankton assemblages have been used to assess grazing rates, particle selectivity, and endocytotic abilities in various marine species, from single-celled organisms to higher invertebrates (Cucci et al. 1989). For instance, Christaki et al. (1999) compared the consumption of two picoplankters *Synechococcus* and *Prochlorococcus* by an algivorous ciliate, *Strombidium sulcatum*, and a bacterivorous ciliate, *Uronema* sp., using FCM. Jonker et al. (1995) studied grazing by *Daphnia* on size classes of phytoplankton

in a freshwater lake. If the grazing organisms are not too big they themselves can be analyzed by FCM to quantify the total number of ingested particles per individual directly, the amount of ingested fluorescence being proportional to the number of prey items grazed, at least during the initial ingestion phases.

FCM can be used to study other aquatic microbial processes through the capability of certain instruments to sort populations from samples and conduct rate-related studies (Li 1994). The major processes associated with plankton involve the cycling of elements such as carbon and sulfur, nitrogen, phosphorus, and iron (Mills et al. 2004). Abundance data can be used to determine the contribution of particular groups to total primary production. FCM sorting can also be used to directly measure group-specific rates of primary production in picoplanktonic populations (Li 1994; Zubkov et al. 2003). Certain phytoplankton groups are responsible for the emission of approximately 20–50 million tonnes of sulfur into the atmosphere annually through their production of sulfur compounds like dimethylsulphoniopropionate (DMSP). FCM can be used to quantify the cellular concentrations of DMSP in plankton and can then help in our understanding of the passage and transformation of these compounds through the marine foodweb (Archer et al. 2001; Burkill et al. 2002). Nutrient limitation and utilization by phytoplankton and their effect on other processes can also be better understood using FCM to quantify specific components of the plankton community and to investigate their response to different nutrient regimes, both in terms of abundance and changes in optical properties.

13.6.2

Cell-related Processes and Functioning

There are various ways of addressing the internal physiology and the health status of phytoplankton cells using FCM. In the case of mixed communities, differences in the physiology of the co-occurring species (inter-specific variations) can be used to explain species dominance and/or succession. However, since FCM measures each single cell separately, it also offers the possibility of studying differences within a single population (intra-specific variation). In particular, the last point addresses a classical concept that a population of cells would be uniform in their response.

Insight into the cell physiology assists us in understanding species and ecosystem dynamics. The most simple of all physiological responses is the autofluorescence signal of chlorophyll *a* in phytoplankton. The pigment properties respond not only to changes in light conditions but also to nutrients as well as to trace metals such as iron. Cell cycle analysis after the measurement of DNA content (see also Chapter 14) has been successfully used to assess *in situ* growth rates of a single species. Recently, Veldhuis and Wassman (2005) applied the method to different subpopulations of the colony forming phytoplankton *Phaeocystis* (Prymnesiophyceae).

Besides probing the cell cycle by measuring cellular DNA content with fluorescent DNA stains, the mean cell size and diel variations in light scattering proper-

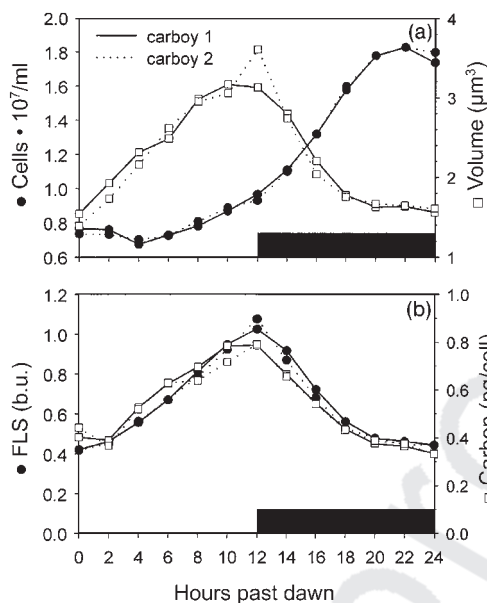


Fig. 13.7 Time series of (a) cell concentration and cell volume, and (b) flow cytometric forward light scatter (FLS, bead units) and carbon per cell for replicate carboys of *Micromonas pusilla* cultures. The black bar denotes when the lights were off in the incubator (12–24 h past dawn).

ties can be followed (for the analysis of DNA content see also Chapter 12). Durand et al. (2002) conducted experiments with mono-cultures of *Micromonas* (Prymnesiophyceae) and found that the cells increased in cell size and carbon content during the light period and then decreased in size with cell division during the dark period. FCM forward and side light scattering followed the same diel pattern, as did cross sections for attenuation, scattering and absorption (Fig. 13.7). The refractive index, calculated using the anomalous diffraction approximation, did not show any significant trend with the light/dark cycle. Since the single-cell measurements of forward light scattering were strongly correlated with independent measurements of cell volume and attenuation cross section, this data set could help provide calibrations for the use of FCM measurements of similar phytoplankton made at sea, to estimate cell size and growth rates and contributions to bulk optical properties such as beam attenuation.

The use of fluorescent oligonucleotide probes targeting the small subunit of ribosomal RNA (18S or 16S rRNA) could provide population-specific proxies of growth rate in the natural environment. Biegala et al. (2003) demonstrated on different picoeukaryote cultures that the fluorescence of cells tagged with probes changed significantly from exponential to stationary phase. In the natural environment similar changes of fluorescence was observed within specifically tagged

picoplanktonic populations. More research needs to be done in that area to help to develop such species-specific growth rate proxies.

Another approach is to address the general physiological status of cells by loading them with a non-fluorescent substrate which, after enzymatic hydrolysis, results in a bright fluorescent product. It is, however, crucial that the end-products after cleavage be retained inside the cell, which is not always the case. Also, the status of cell membranes appears to be a useful tool to determine the viability of cells. Veldhuis et al. (2001) determined the viability of different phytoplankton species based on a staining procedure using the nucleic acid dye SYTOX Green. The assay is based on the particular characteristic of this stain that it can only penetrate cells with a compromised plasma membrane but cannot penetrate membranes of living cells (Roth et al. 1997). Thus the DNA of viable phytoplankton cells will not stain whereas the DNA of non-viable cells will show a bright green fluorescence.

Many cellular process studies have investigated phytoplankton cultures as well as field samples. During the past few years several of these FCM–cell physiology assays have been used in nutrient limitation studies, for toxicity tests, to determine the role of viruses and even in ships' ballast water research. These studies have altered our perceptions about the apparent homogeneity of populations, not only in the field but even in algal mono-cultures.

FCM systems may play an important role in the assessment of phenomena studied in the field of biological oceanography as well as ecophysiology of plankton. Ecosystem dynamics are driven by the interplay between the dynamics of the surface ocean mixed layer and the depth of light penetration. A “cascade of turbulence phenomena” leads to a range of mixing scales with photosynthesis depending on irradiance, photo-inhibition and photo-adaptation, temperature, nutrients, phenotypic, and genotypic variance. FCM data can be included in a larger data set alongside environmental parameters in order to estimate eco-physiological key processes in the upper water column. A continuous and relevant process with respect to photosynthesis is light acclimation of phytoplankton. FCM can be used to study photo-acclimation with respect to light variations at different scales of time and space. *In situ* studies, as well as controlled environments (laboratory and *in situ*-simulated environments), were reported by Brunet et al. (2003), in relation to vertical water movements, and Dusenberry et al. (2001). Other topics studied are mesoscale features of phytoplankton and planktonic bacteria in coastal areas induced by external water masses (Casotti et al. 2000). **■AQ1■** Dignum (2001) developed a straightforward method to determine phosphate availability for individual algae. This technique was successfully applied in 2001 in the Loosdrecht Lakes and uses the response that phytoplankton cells themselves show when there is a lack of phosphate, by conversion of specific enzyme activity into a localized fluorescent signal. The fluorescence of individual algae is measured on a flow cytometer. The full benefit of such diagnostic tools requires integration of field data with experimental assessment of relevant population characteristics, and prediction of the complex interactions between algae and their environment.

Different types of lakes are expected to show seasonal and local variations in the nutrient status.

13.6.3

Plankton Abundance Patterns in the Sea: Indicators of Change

FCM is well suited to the examination of multiscale patterns in plankton ecology. Since measurements are easily taken at short time scales and at small spatial scales, and since environmental monitoring programs can be sustained for long time periods over large geographic regions, possibilities exist to scale microbial interactions to regional and global phenomena (Li et al. 2006a, 2006b). Long-term studies of phytoplankton abundance and diversity indicate that directional changes have occurred on both large (Richardson and Schoeman 2004) and small spatial scales (Ribera d'Alcalà et al. 2004) in many places. Inherently, biological populations undergo strong natural fluctuations. The ability to discern secular change from natural variability depends on a time series of relevant measurements made at an appropriate frequency for an extended period of time over a wide area. Because FCM is designed for rapid quantitative screening of fluorescence and size-related characteristics of single cells, it becomes feasible to map selected aquatic microbes at high resolution in both space and time, limited only by the number of water samples recovered in hydrographic surveys. Yet it remains true that no map of a plankton variable derived from shipboard surveys can capture all the variance at relevant space and time scales. However, statistical examination of extremely large data sets provided by FCM can reveal general phenomenological patterns that indicate intrinsic, evolutionary or extrinsic constraints on variation. In large data sets, thousands of individual contingent case histories can be subsumed to provide a detail-free holistic view (Li 2002). Patterns of abundance for cells of different sizes examined in relation to attributes such as autotrophic biomass, primary production and water column stability indicate the possible reactions that phytoplankton communities may adopt in the face of environmental change.

13.7

Marine Optics and Flow Cytometry

In situ cytometer systems open up new possibilities for establishing a link between measurements of single particle optics and bulk inherent optical properties, based on assessing populations of marine particles. When flow cytometers were first introduced to the marine sciences, instruments were taken to sea in dedicated container laboratories with separate industrial cooling sections (Tarran and Burkill 1992). However, the introduction of air-cooled argon lasers soon led to compact bench-top designs, and it was anticipated that FCM would make a major contribution to our understanding of the nature and dynamics of particle suspensions in the sea (Demers 1991). The potential value of the technique was demon-

strated by the detection of ubiquitous and previously unknown populations of marine prochlorophytes (Chisolm et al. 1988). Early studies of single-cell phytoplankton optics included estimates of absorption and cross-sections (Perry and Porter 1989), refractive indices (Spinrad and Brown 1986), forward scattering patterns (Cunningham and Buonaccorsi 1992), and anomalous scattering from cyanobacteria (Dubelaar et al. 1987). However, from the point of view of marine optics, much of the research momentum seems to have been lost in recent years.

The few research groups currently carrying out FCM at sea are concentrating on problems in microbiology (Sieracki et al. 1995), picoplankton physiology (Boelen et al. 2000), and grazing by microheterotrophs (Zubkov et al. 2000). Recent attempts to draw up a database for the optical properties of marine particles (Stramski and Mobley 1997) show that there is an urgent need for information on natural particle suspensions. The importance of establishing a link between measurements of single particle optics and bulk inherent optical properties was first discussed by Morel (1991), but remarkably little progress has been made since. We anticipate that the availability of *in situ* FCM systems will open up new possibilities for studying populations of marine particles *in situ*, and there are a number of identifiable topics of current ecological interest which would benefit from the technique. These include detecting thin layers of heterotrophic activity which are believed to exist close to major pycnoclines, monitoring the physiological status of sinking algal blooms, investigating variations in diatom chain length under different physical conditions (e.g. turbulence regimes), and counting phytoplankton cells in waters which bear a heavy burden of other suspended particles. An example of the potential use of *in situ* flow cytometers has already been described earlier in this chapter and involved a CytoSub flow cytometer, with additional marine optics instrumentation onboard an AUV, passing through a coccolithophore bloom. The data generated by CytoSub along the transect corresponded well with reference measurements from other marine optics instrumentation (Fig. 13.8).

The main potential contribution to ocean optics is the possibility of accurately characterizing mixed particle assemblages in terms of the numerical proportions of different classes of the materials and of the distribution of sizes within each class. This information should provide new insight into variations in volume scattering functions, particularly in coastal waters. Wider application of FCM to the determination of single particle optical characteristics may help advance our understanding of the characteristics of mixed particle suspensions and hence, in an interesting contrast of physical scales, aid in the interpretation of satellite measurements of remote sensing reflectance.

13.8 Future Perspectives

The hydrological and ecological composition of our surface waters is intrinsically patchy and dynamic. Therefore the determination of “critical scales”, and deploy-

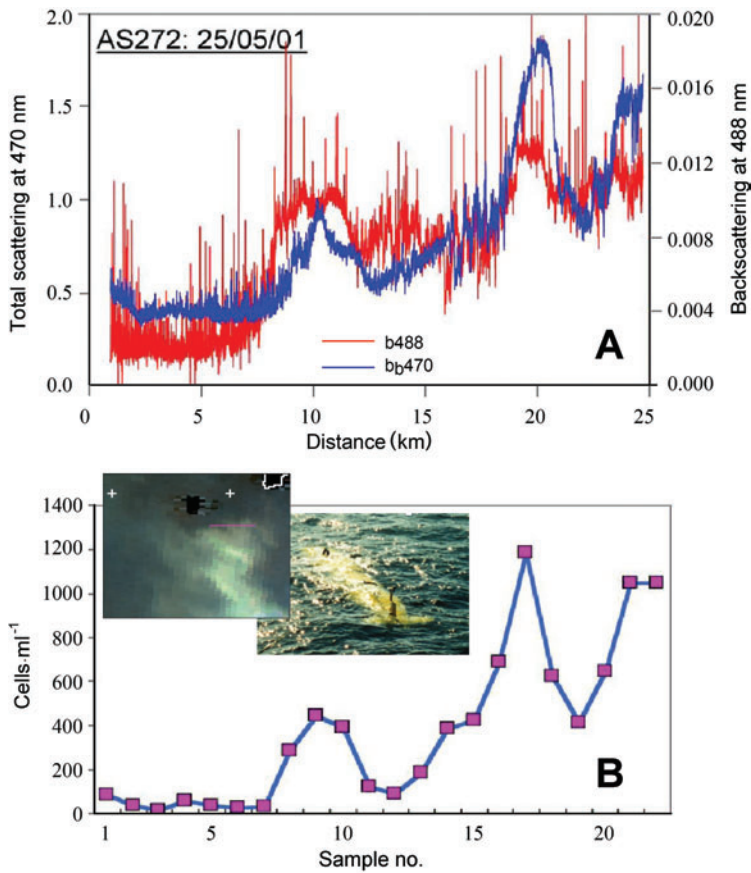


Fig. 13.8 Analysis of coccolithophores (Haptophyta) bloom SE of Isles of Scilly, SW England, 24 May 2001. (a) Ac9 (flow-through absorption attenuation meter, WET labs, Philomath, OR, USA) total scattering (left scale) and Hydroscat (multi-wavelength optical backscattering sensor, HOBI Labs, Tucson, AZ, USA) back-scattering (right scale) transects from Autosub (autonomous underwater vehicle, Southampton Oceanography Center and NERC). (b) *Emiliana huxleyi* cell counts from CytoSub (submersible flow cytometer, CytoBuoy b.v.) traveling in the Autosub submarine. Left insert, SeaWiFS (Sea-viewing Wide Field-of-view, NASA) RGB image of coccolithophores bloom (Remote Sensing Data Analysis Service, PML, UK), the purple line marking the Autosub transect. Right insert, the Autosub on a mission.

ing the technology to sample at these required scales often leads to the discovery of new kinds of patterns or phenomena. This information may lead us to abandon old ideas, ask new questions, and revolutionize scientific paradigms (Donaghay and Osborn 1997). Perhaps the most exciting perspective of the advent of modern, easy-to-use and especially the aquatic flow cytometers is our increasing ability to analyze microbial assemblages at their relevant critical scales in time and space. Integration with other technologies seems to be the way forward

(Babin et al. 2005). Further development of dedicated combinations of instrumentation, pre-processing protocols and data analysis algorithms as well as merging data from different scales from microscope to satellite will allow a shift in applications towards powerful solutions for regular monitoring and control situations as well as *protection and early warning* applications.

The potential applications are numerous, in which FCM can be used to detect phytoplankton and other organisms, such as bacteria in industrial and regulatory situations. Harmful algal blooms and pathogenic bacteria are a constant threat in the aquaculture industry, drinking water industry and for bathing and recreational waters. Regulations are becoming more stringent (e.g. EU Bathing Waters Directive, EU Shellfish Waters Directive) and regulatory authorities are looking for more rapid techniques to provide early warnings of potential pollution incidents and also to monitor the disappearance pollution so that the situation can be declared safe again. Flow cytometry, either as a stand-alone technique or in conjunction with other techniques, such as fluorescent species-specific probes, is well placed to provide rapid solutions for regulatory authorities. Autonomous and high frequency sampling may provide detailed information about community composition and abundance. This would make it easy to detect harmful species at pre-bloom concentrations and also to assess physiological states and viability. This information is crucial for making rapid decisions regarding harvesting aquaculture stock, take other appropriate measures to protect production or, in the case of bathing waters, informing the public about safety issues on beaches and around recreational lakes.

FCM could also be used to detect pollution by herbicides and other toxins (Readman et al. 2004) by analyzing their effects on phytoplankton abundance and cellular characteristics (Fig. 13.9). Using a sufficiently high sampling frequency enables the detection of sudden changes. For example, the fluorescence increase shown in Fig. 13.9b indicates an initial blocking of the photo-system followed by cell death. FCM combined with phytoplankton as bio-indicators could therefore be used to provide an “aqua-alarm” function.

Another very important potential use for FCM concerns monitoring ballast waters from ships for invasive marine species. Invasive marine species have been identified as one of the greatest threats to the world's oceans. These species enter into their new environments via ships' ballast water, attached to ships' hulls and via other vectors. Ships use ballast water to maintain their balance, allowing for differences in cargo-weight and bunker fuel oil. To minimize the risks of ballast water transport, the International Maritime Organization (IMO) has adopted a convention for ballast water management on board ships with discharge standards for treated ballast water that should be complied with (Anonymous 2004). This can be done by ballast water exchange in open sea or by using ballast water treatment installations. The standards include limits for discharge of phytoplankton-sized organisms, as well as pathogens. FCM technology (Veldhuis et al. 2006) can be used for rapid, online monitoring of the performance of ballast water treatment installations, including sizing, enumerating, and assessing the viability of the individual organisms.

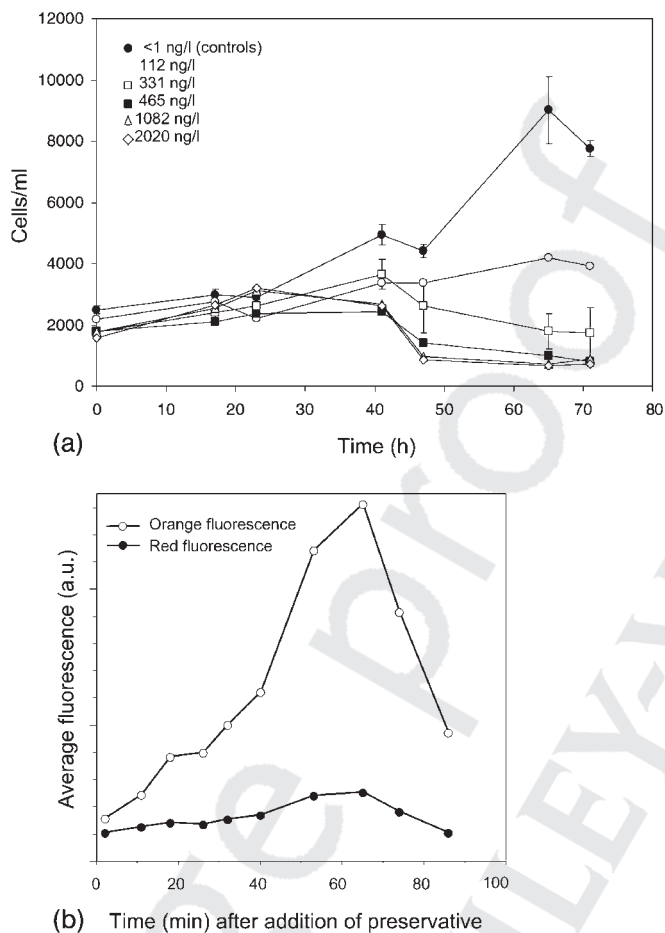


Fig. 13.9 (a) Cell numbers as measured by flow cytometry, of eukaryotic phytoplankton exposed to different concentrations of Irgarol® 1051, a phytocide and photosynthesis blocker, during a 72-h period (from Readman et al. 2004). (b) Average autofluorescence of Irgarol®-treated *Chroomonas salina* cells in culture (Plymouth Marine Laboratory) measured by flow cytometry at 532 nm excitation.

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