SCI. MAR., 64 (2): 135-156 SCIENTIA MARINA 2000

AQUATIC FLOW CYTOMETRY: ACHIEVEMENTS AND PROSPECTS. *M. RECKERMANN and F. COLIJN (eds.)*

Flow cytometry as a tool for the study of phytoplankton*

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SUMMARY: An overview is presented on flow cytometry as a tool for counting, analysis and identification of phytoplankton species and groups. The paper covers basics on the analysis technique and instrumentation such as the measuring principle, the type of instrument, limitations and pitfalls with phytoplankton samples and sample handling and preprocessing. Possibilities of the measured entities are discussed, roughly divided in light scatter and related parameters, the endogenous fluorescence and exogenous fluorescence, followed by a discussion on the actual applications such as phytoplankton abundance analysis, ecology and physiology research and monitoring of particle size and biomass. In addition to a limited literature review, we tried to assess how flow cytometry is used in routine laboratory practice and monitoring operations. Therefore, a questionnaire was sent out via email to 47 scientists at 43 institutes known to us as involved in flow cytometric analysis of phytoplankton. In total, 19 scientists responded. Specific survey results are included in italic print whereas some more general answers were integrated in the overview.

Key words: flow cytometry, phytoplankton, marine, fresh water, monitoring, counting, identification, fluorescence, particle analysis.

INTRODUCTION

The aquatic environment is subject to dynamic processes on widely varying time and space scales. The scale of the smallest independent biological unit, the cell, remains a key scale for interpretation and calibration of data. Although traditional microscopical analysis is unsurpassed regarding species identification power, there are drawbacks in terms of enumeration, quantitative character and speed. Flow cytometry allows fast counting and optical analysis of individual particles, although with less detailed species discrimination. Li (1995) for instance showed with flow cytometry that *Prochlorococcus* spp. comprised 78% of the cells of central North Atlantic Ocean ultraphytoplankton, representing 28% of total fluorescence, a measure of chlorophyll biomass, and about 11% of total light scattering, being a measure of carbon biomass. In addition, the high number of cells analysed by flow cytometry permits more statistically significant results, important for instance to study population and community structures, as shown also by Gisselson *et al*. (1999) who used cell cycle analysis to estimate *in situ* gross growth rates. The first flow cytometric studies in aquatic sciences were published over a decade ago (Paau *et al*., 1978, 1979; Trask *et al*., 1982; Yentsch *et al*., 1983a). Milestones were the special issue of

*Received June 14, 1999. Accepted March 24, 2000.

Cytometry (Yentsch and Horan, 1989) and the NATO Advanced Study Institute on Individual Cell and Particle Analysis in Oceanography (Demers, 1991). Overviews were presented by Yentsch (1990) and Olson *et al*. (1991). Samples containing fresh water or coastal water phytoplankton populations are much more heterogeneous in terms of size, morphology and concentration as compared to the blood cell samples for which flow cytometers were originally developed. Aquatic field samples are analysed quite regularly though in the practice of aquatic science, mainly using commercially available standard equipment, and occasionally using experimental instrumentation. Particles in the open oceans are usually small, allowing oceanographers to use flow cytometers without many problems, which led to the discovery of *Prochlorococcus* (Chisholm *et al*., 1988), and the determination of the "smallest eukaryotic organism" (Courties *et al*., 1994). Detection of 'new' species or groups using flow cytometry still occurs (Corzo *et al*., 1999). Parallel to the increasing application to algae, and with the advent of more sensitive optics, flow cytometers were also used to measure aquatic bacteria (Button and Robertson, 1990), contributing to the awareness of the importance of aquatic bacteria over the past years (Azam and Smith, 1991). Robertson and Button (1989) developed procedures for characterizing aquatic bacteria according to population, cell size, and apparent DNA content. Endo *et al*. (1997) described a rapid flow cytometry technique based on fluorescein diacetate to distinguish between viable cells and dead cells for various sea water bacteria. Flow and image cytometry were used by Sieracki *et al*. (1995) to demonstrate overestimation of heterotrophic bacteria in the Sargasso Sea by standard microscopical technique. The most recent achievements in terms of detection of small particles are the enumeration of aquatic viruses (Marie *et al*. 1999), making FCM a potentially useful tool for studies of the structure and dynamics of virus populations in natural waters and basic virus-host cell interactions (Brussaard *et al*. 1999).

All institutes of the survey are or were using flow cytometry for the analysis of field samples. Twelve institutes say they use, used or will use flow cytometry on a routine basis. The 9 institutes who are currently doing regular analysis of field stations have sampling strategies varying from weekly to yearly analysis, depending on water type (estuarine > shelf > oceanic), logistics (close to the lab > far away) or otherwise. The combined numbers of stations covered are: 17 stations at a weekly or biweekly basis, 13 stations at a monthly or bimonthly interval and more than 20 stations once per season or year. Sample collection varies between 1 and 10 depths sampled per station. The total number of field samples processed annually varies from about 50 to about 1000 per laboratory. Besides daily laboratory operation, 7 institutes employ the flow cytometers on research cruises on a more or less regular basis. During the cruises, typical strategies are daily analysis of depth profiles, up to sampling every few hours.

TECHNOLOGY

Measuring principle

Figure 1 is a schematic presentation of the measuring principle and data processing of a flow cytometer. Flow cytometers measure light scatter and fluorescence of particles passing a zone of intense illumination, carried and centred inside a high speed water jet, free flowing in air or in a quartz flow cuvette. Most cytometers use a laser for illumination. Laser light is monochromatic, with ultraviolet, visible or near infrared lines. Small air cooled argon lasers delivering 488 and 515 nm beams of up to about 100 mW are most commonly used. The cells are pumped in a single file through the analysis point at typically 1,000 cells or more per second, with a practical analysis speed of 1-5 minutes per sample. The successive scattering and fluorescence signals generated by each passing particle are detected by photomultiplier tubes or photodiodes. The detection sensitivity is sufficient to analyse submicron particles. The electronics interface converts these raw signals into correlated digital data, stored on disc for data analysis and presentation as distributions (univariate) or multivariate scatterplots or grey/colour maps. Instruments may have a sorting device, allowing the physical separation of selected cells from the main stream during analysis (Reckermann, 2000). Wallner *et al*. (1997) sorted bacteria from lake water and sediment based on differences in light scattering, DNA content, and/or using rRNA-targeted oligonucleotide probes. The cells of a single population give similar results, showing as a single peak in a univariate distribution, or a close group of data points in a multidimensional scatter plot. In addition to the instrumental properties, it is the biological variance, such as differences in cell size, life cycle, pigment content etc.,

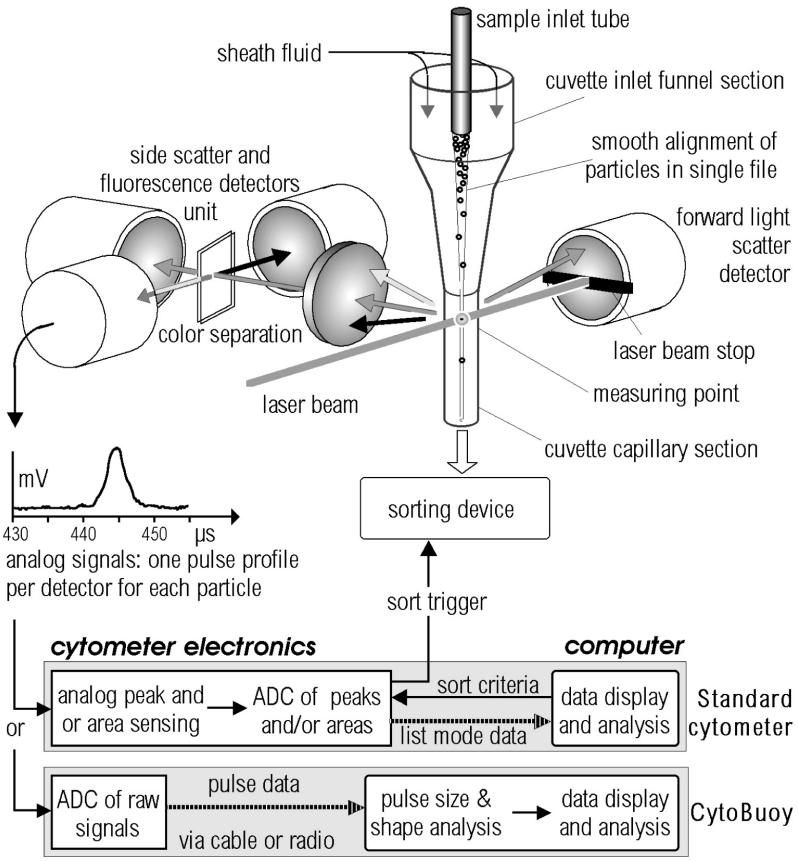

FIG. 1. – Schematic drawing of the flow cytometer operating principle and signal processing. The sample is injected in a sheath fluid that funnels it into a thread, so thin that the particles are well separated, intersecting a focussed laser beam one by one at high flow speed. Here, each particle emits a short (typically a few microseconds) flux of photons by scattering or fluorescing. Photodetectors convert those into electrical pulses. Small particles give similar, gaussian shaped pulses, following the distribution of light in the laser focus. From these signals (top electronics panel), either the pulse maximum or the pulse area is sensed with analog electronics, subsequently digitized and stored on computer disc in so-called list mode data files. In these files each detected particle is listed with its indiviual pulse values, one for each detector. The directly available listmode data per cell may also be used to control a sorting module for the real-time physical selection (flow sorting) of particles downstream of the laser focus. The shape and length of big diatoms and filaments dominate their detector pulses, impairing analog electronic processing. This requires direct digitization of the detector output (lower panel) to obtain correct pulse area (integral) values and to allow any other type of pulse analysis in principle, as applied in the CytoBuoy instrument (Dubelaar *et al.,* 1999, Dubelaar and Gerritzen, this volume).

that causes the variance seen as the width of the peaks or clusters (Campbell *et al*., 1989a, b, c). With mixed cultures or field samples, more clusters appear, more or less separated, representing groups with different optical properties. Figure 2 shows a typical bivariate plot of a sample containing several species. The data points from a cluster can be selected to yield distributions of physiological properties of the cells belonging to this group (Li, 1990; Demers *et al*., 1992). Exploration of data analysis tools such as multivariate curve-fitting and diversity indices is important in this respect. The discriminating power of the analysis increases if more independent optical properties of the particles are measured, or if specifically binding fluorescent probes are used. This is important to allow classification of the particles.Whereas in the biomedical field, the cytometrist is faced with perhaps four to six or so cell types to differentiate, in marine waters the numbers of cell types are typically up to an order of magnitude greater. The development of procedures for automated data analysis is crucial. Recent advances achieved in neural network computing are promising (Smits *et al*., 1992; Balfoort *et al*., 1992b; Frankel *et al*., 1989, 1996; Wilkins *et al*., 1996; Jonker *et al*., 2000).

Commercial instruments

The number of flow cytometers world wide is probably around the 10.000 figure. Designed for analysis and sorting of mammalian cells, by far the

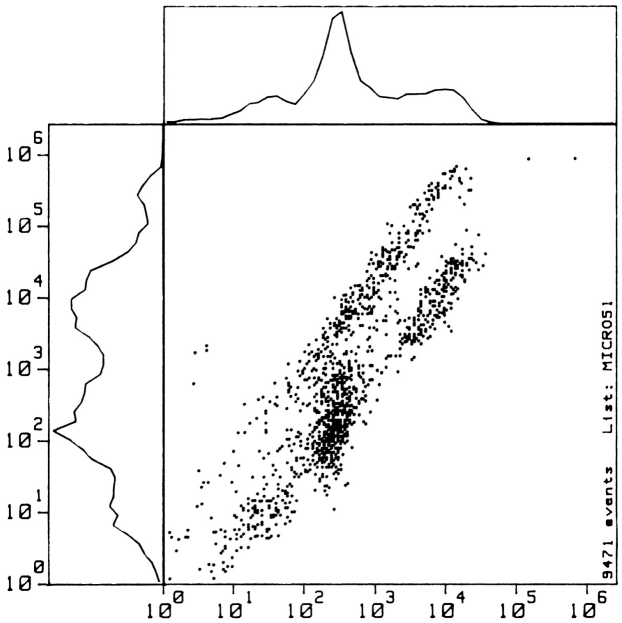

FIG. 2. – An example of a bivariate scatter plot, with corresponding univariate frequency distributions, of a fresh water field sample containing single cells and some colony forming cyanobacterial species, measured with an OPA flow cytometer. Vertical axis = side scatter; horizontal axis = forward scatter. Each dot in the scatter plot represents an individual particle. The frequency distributions show the number of particles versus their measured forward or side scatter value (horizontal and vertical distribution respectively, numbers normalized to fit the frame).

largest part, say about 95% of the instruments are used in biomedical applications. The rest are employed in a variety of fields such as pharmaceutical industry, food and beverages industry, diary industry, botany, marine science, limnology and drinking water industry. Becton Dickinson (BD Immunocytometry Systems, San Jose, CA, USA) and Coulter (Beckman Coulter Inc., Fullerton, CA, USA) hold the major market shares with their FACS and EPICS flow cytometry systems. Smaller competitors are Partec GmbH (Münster, Germany) with the PAS multiparameter laser and arc lamp flow cytometry system, Cytomation Inc. (Fort Collins, CO, USA) with the MoFlo, a top grade 3 laser, 12 parameter high speed research sorter, and Optoflow AS (Oslo, Norway), a relative newcomer aiming specifically for the low cost side with the small, portable all solid state Microcyte flow cytometer. No recent information was found on the Cytoron Absolute flow cytometer (Ortho-Clinical Diagnostics, Raritan, NJ, USA). BioRad discontinued their arc lamp based Bryte HS flow cytometer. Not a flow cytometer, but very interesting is the LSC microscope-slide based laser scanning cytometer from Compucyte (CompuCyte Corporation, Cambridge, MA, USA). None of these instruments is particularly designed for marine and aquatic research. It seems in practice that aquatic scientists have to explore changing the main characteristics of their standard instruments from medicine to plankton research, e.g. to find the optimum optical filters and combinations and to enhance the signal/noise-ratio as far as possible. However, even if a cheap and useful instrument would generate a boost in flow cytometer sales in the aquatic research field, the resulting market still would remain an order of magnitude smaller as compared with the biomedical market. It is not likely therefore that a dedicated instrument for the aquatic market will be released soon by these existing manufacturers.

The answers on instruments used in the survey concerned in total 30 instruments more or less regularly used by the institutes for phytoplankton analyses. Most frequently used are the instruments from Becton Dickinson (14 instruments with 5 FACSort and 4 FACS Calibur instruments) and Coulter Electronics (8 instruments). Some no longer built instruments are still in use such as a Bruker instrument (related successor by Bio-Rad now). Some in-home modified machines and completely dedicated instruments are used (Table 1). About 10 institutes operate more instruments, with a relative new instrument for routine work and cruises and an older instrument still in use for back-up, and experimental work or modifications.

Limitations and pitfalls with phytoplankton samples, dedicated instruments, methodology

Cells may be affected by fluid acceleration, electrical shock and most importantly, light shock in flow cytometers, possibly influencing subsequent analyses (Rivkin *et al*., 1986; Haugen *et al*., 1987). The instrument performance in turn may be impaired by the specific properties of aquatic samples. Low concentrations may require either preconcentration which deteriorates the sample composition, or pushing the sample flow rate to the max, leading to less accurate measurements.

Almost all participants of the survey considered the small sample volumes processed by flow cytometers a bottleneck. With low cell concentrations in natural samples, relatively large sample volumes should be analysed in order to get an acceptable statistical count. This is time consuming in standard machines: 2 ml = around 25 minutes using the FACSCalibur. In addition, as diversity increases, the

TABLE 1. Instruments and set-up used among the survey group.

#	manufacturer	type	illumination bands				fluoresc. detector bands					nozzle cuvette	sorting	other
			UV	blue	green	red	blue	green	yel	oran	red			
			mW	mW	mW	mW	nm	nm	nm	nm	nm	μm		
4	Becton Dickinson	FACS Calibur		15				x		x	x		x	
5	Becton Dickinson	FACSort		x				x		x	x		x	
1	Becton Dickinson	FACS Analyzer	high pressure Hg lamp						x		x			ERS
2	Becton Dickinson	FACScan		15				x	x		x			
1	Becton Dickinson	FACScan	in-home modified for high sensitivity analysis											
1	Becton Dickinson	FACS Vantage	50	150		35		x	x		x	100–400	<200 μm	
1	Bruker Spectrospin	Argus	arc lamp instrument, jet-on-open-surface instrument											
1	Coulter Electronics	Epics Elite	100	30			x	x		x	x	250	<40 μm	
1	Coulter Electronics	Epics Elite ESP	50	200										
2	Coulter Electronics	Epics XL		15										
1	Coulter Electronics	Epics 753	in-home modified for high sensitivity analysis											
1	Coulter Electronics	Epics V	or	or	or								high speed	
1	Coulter Electronics	Epics V	in-home modified for high sensitivity or high sample flow settings											
1	Coulter Electronics	Epics 541	or	or	or		2 fluorescence detectors						autosampler	
1	Ortho Diagn. Instr.	Cytoron Abs.		15				x		x	x			
1	Max-Planck Inst.	Fluvo II	mercury-lamp				2 fluorescence detectors							ERS
1	PARTEC	PAS-III			30				x	x	x			
1	PARTEC	PAS-III	lamp	150				x		x	x		optional	ERS
1	TNO - NL	OPA	Exp. instr. for large size range linear analysis									1000		
2	EC MAST-3 group	Eur-OPA	OPA type instrument with various experimental options											

total nr. of instruments in mini-survey group

number of measured particles per sample has to increase accordingly for proper statistical analysis of the less abundant species. Typically the cells larger than 15 - 20µm, often comprising the bulk of biomass in eutrophic coastal environments, are relatively rare.

Standard instruments have limited particle size ranges, with upper limits of typically 30 to 150 μm. Large(r) particles result in system clogging or 'merely' loss of data quality. Examples of the latter are selectivity against large particles owing to small orifices and tubes in the fluid system or 'dead spaces' where large particles settle out. Particles may exceed the size of the optical sensing zone of the instrument, their electronic signals may exceed the proper range, a source of artifacts such as peak height sensing with particles longer than the height of the laser focus (Peeters *et al.*, 1989) or time-of-flight analysis of filamentous species. Big particles slow down the fluid in a cuvette (about 20% for a particle half the channel diameter). Long fragile particles may break upon entering the flow channel, where velocity gradients are highest. Other limitations are insufficient analytical power for the wealth of cell and colony shapes, or insufficient sensitivity for the small aquatic microorganisms. A very high concentration of small particles, including those below the detection limit, may cause an upward drift of the electronic 'background signal'. This may occur with flow cytometry of sea water viruses at concentrations of for instance 10 billion per liter (Fuhrman, 1999). Even a 100 times diluted, their counting rate would be between thirty and a hundred thousand per second, causing a higher background signal that could decrease the detection probability for the smallest particles, and raise the relative counts of virus doublets as reported by Marie *et al.* 1999.

Olson *et al.* (1983) and Cunningham (1990a) constructed low-cost flow cytometers for phytoplankton analysis. High-sensitivity flow cytometers for studying picoplankton were developed by Frankel *et al.* (1990) and Dusenberry and Frankel (1994). Hüller *et al.* (1991) reported on a macro flow planktometer for analysis of large marine plankton organisms (>100 μm). The optical plankton analyser (OPA) was

developed for field samples (Balfoort *et al*., 1992a; Dubelaar *et al*., 1989) containing single cells and colonies, including aggregates and filaments with lengths over a millimetre, being measured by the OPA with fair linearity (Dubelaar and van der Reijden, 1995). In the subsequent EurOPA project (Dubelaar *et al*., 1995b), a more versatile instrument was developed with a photodetector array probing diffracted light, a pulse profile acquisition module (Cunningham, 1990b), a cytometric imaging device (Wietzorrek, 1994; Kachel and Wietzorrek, 2000) and a sorter system. Cavender-Bares *et al*. (1998) developed a dual sheath flow cytometer for shipboard analyses to cope with widely varying and very low concentrations of phytoplankton in the oligotrophic oceans. Chekalyuk *et al*. (1996) developed a prototype pump-during-probe fluorometry flow cytometer to measure the photosynthetic capacity of cells. None of these special instruments were commercially produced on a significant scale to date. Analysis algorithms for the processing of the measured data were deveoped such as various artificial neural network approaches (Boddy and Morris, 1993; Boddy *et al*., 1994a, b) and multi-variate statistical protocols - the quadratic discriminant analysis being fast and the canonical variate analysis being graphically useful (Carr *et al*., 1994, 1996).

Sample handling and preprocessing

Avoiding particle selectivity and damage to fragile particles is not trivial. Each of the sampling, subsampling, filtration, preservation, concentration, staining, storage and transportation processes are potential sources of bias or variability. Flow cytometry requires small sample volumes, but allows more samples and less sub-sampling to obtain good statistical significance. Pre-filtration of field samples prevents clogging of the instrument flow system; concentration reduces the sample volume. Both easily impair the composition of samples containing different and fragile species. For a tenfold concentration of North Sea samples, Hofstraat *et al*. (1990) successfully applied a combination of sedimentation and upward filtration at low suction head. Bloem *et al*. (1986) examined filtration and centrifugation of heterotrophic nanoflagellates. Centrifugal elutriation provides an alternative cell separation and concentration technique when large numbers of cells are required (Pomponi and Cucci, 1989). Preservation is used to preserve cell integrity and fluorescence properties for periods of months. Although fluorescence gradually decreases in many cases, the preservation may cause an initial short term (minutes to hours) boost in fluorescence by blocking the energy transfer mechanisms in the pigments. Flow cytometers count particles, but one obtains accurate particle concentrations only if i) the sample flow is steady and calibrated and ii) if there is no unknown loss of particle counts by coincidence. Flow cytometer sample systems driven by air pressure may not be as steady and reproducable as volumetric pump driven systems. Coincidence is proportional to the particle concentration and the dead time, occuring if particles are so close in the sample stream that they are measured together (looking like a single particle with double signal intensity), or if the opto-electronic system is still busy processing the signal of the first particle and the second goes undetected: the 'dead time effect'. Particularly with more than one laser, the dead time may be significantly larger depending on the type of electronics. Whereas most aquatic field samples have low particle concentrations from a flow cytometrical perspective, cultures and bloom samples may have to be diluted. In cases of uncertainty, reliable particle counts may be obtained by adding a known amount of fluorescent calibration beads to each sample, and to correct the cell counts for the number of detected beads. The beads can also serve as a signal intensity and/or quality reference.

The survey showed that the general working principle is to try to analyse the samples in as close to natural state as possible. Preconcentration was not mentioned, prefractioning once. Adding calibration beads for concentration and data quality assessment was mentioned once (this was not an explicit question). Preferentially, samples are measured fresh, immediately after sampling. Logistics may make it necessary to store samples, i.e. for a few days (storage at 4° in the dark). This applies for the analyses done during cruises, and samples collected from shore or on short cruises. Small and fragile cells suffer also from these short periods of storage and may disintegrate. Light scatter properties will be influenced, as well as chlorophyll-a fluorescence. Fixatives used are based on what is used in microscopy and include formaldehyde (mentioned 4 times), paraformaldehyde (mentioned 5 times), glutaraldehyde (mentioned 6 times) and sometimes combinations. Lugol was not mentioned since it deteriorates fluorescence. Long time storage predominantly is in liquid N_2, in combination with 1% glutaraldehyde fixation after Vaulot et al. (1989).

This method works well with picoplanktonic populations. Troussellier *et al*. (1995) examined effects on bacterioplankton and picophytoplankton. Larger and more fragile cells can be lost to an important extent however, and show variation of chlorophyll fluorescence. A possibility for improvement would be the addition of cryoprotectant (Lepesteur *et al*. (1993), although both optical properties and cell numbers could not be preserved well. A protocol with 0.1% to 0.5% paraformaldehyde (methanol-free) and storage at 4° was developed at JRC-Ispra (Premazzi *et al*., 1992). Higher concentrations of paraformaldehyde increasingly impaired the results. Staining cells requires extra steps like permeabilization and washing. Recently, detailed protocols for flow cytometric enumeration of phytoplankton, bacteria, and viruses in marine samples (Marie *et al*., 1999), as well as for DNA/RNA analyses of phytoplankton, were published (Marie *et al*., 2000).

MEASURED PARAMETERS

Light scatter

The light scatter of particles is measured parallel to the laser beam: the forward or low angle scattering, and perpendicular to this: the side scattering. Simple dependence on for instance particle diameter or volume is restricted to limited classes and size ranges of particles. The highest scatter intensities are at small (low) scattering angles. Significant differences exist between small particles as for instance bacteria and larger particles as for instance ciliates. The intensity of the light scattered by the bacteria drops 3 orders of magnitude with increasing angle, whereas the light scattered by the ciliates drops 6 orders of magnitude. The measured forward light scatter depends mostly on the overall cell cross section and not so much on smaller structures, whereas the side scatter is dominated by the small internal and external structures of the particles. Internal micro structures such as light absorbing pigments and intracellular gas vacuoles may have significant effects on the forward light scatter signal, however, caused by their effect on the global refractive index of the small and medium sized cells (Dubelaar *et al*., 1987). The side scatter may yield the most straightforward relation (proportional to particle cross section) for particles of low refractive index, sized from about 1μm upward (Morel, 1991), but is known to be very sensitive to small cellular structures which cause large variation in the data. Whereas forward light scatter is a good measure for cell volume with very small particles (<0.6 μm^3) as shown by Koch *et al*. (1996), the forward light scatter is linearly proportional to cellular cross section only for optically large cells (tens of microns diameter and/or highly absorbing), and shows a fluctuating behaviour at intermediate sizes. In the EC MAST project AIMS (developing flow cytometry technology for identification of microbial cell populations and determination of their cellular characteristics- Geider *et al*., 1998; Jonker *et al.,* 2000), algorithms are being developed to translate flow cytometric light scatter signals to size spectra. Under conditions, size and refractive index of marine particles can be measured (Ackleson and Spinrad, 1988, Spinrad and Brown, 1986). Dilution (osmosis), chemical fixation and/or staining as well as cell damage cause changes in forward light scatter signatures (Ackleson *et al*., 1988; Navaluna *et al*., 1989). Ratios of intensities at different angles can be used for sizing bacteria (Koch, 1986). With azimuthally resolved forward light scatter measurements, typical cell shape information may be obtained (Buonaccorsi and Cunningham, 1990; Cunningham and Buonaccorsi, 1992; Forrest, 1985; Premazzi *et al*., 1989). Smart wiring of a 25 pixel photodiode array, reduces to only 4 measured numbers per particle for symmetry and size information (Dubelaar *et al*., 1995b). As the laser light is linearly polarized, depolarization measurements can be implemented also relatively simply, to probe isotropic cell structures. Olson *et al*. (1989) used polarization properties of forward scattered light in addition to other parameters to discriminate eukaryotic phytoplankton cell types. Particularly, the coccolithophores depolarized forward scattered light. Direct measurements of particle absorption are very difficult with flow cytometers, and no such possibilities were reported. Beam attenuation (axial light loss) can be measured with flow cytometers, but this parameter is dominated by light scatter (Eisert, 1979). Many flow cytometers measure the duration of the pulses, which is a good measure for particle length if scatter pulses are used. Electrical resistance sizing (Coulter volume) is optional on a few instruments.

Endogenous fluorescence

Pigment fluorescence, the major component of endogenous fluorescence, is used for quantification of photosynthetic capacity, biomass and cell size

(Chisholm, 1992), and identification of cellular pigment composition type. Absorbed photons generate higher excitation stages in the chlorophyll antenna pigments, from which photosynthesis is driven or by-products are generated such as heat or emission of lower energy light, fluorescence. One of the key problems in fluorescent diagnostics of photosynthetic organisms is to distinguish the contributions of constant and variable fluorescence components, because the first one contains information on the efficiency of exciton migration through the light harvesting antenna, while the second one reflects the state of PS II reaction centres and the electron-transport chain (Chekalyuk *et al*., 1992). At low light conditions, photosynthesis competes efficiently with fluorescence and fluorescence is low (constant fluorescence). The light intensity in flow cytometers is very high, but the passage time of the particles is extremely short (a few microseconds). The question is to what extent the variable fluorescence rises during the short passage time of the cell through the laser beam. With double or triple beam cytometers, the cell has to travel some time between the laser beams, and effects may be quite different at the down stream laser foci. Studies (Ashcroft *et al*., 1986; Neale *et al*., 1989; Xu *et al*., 1990) on flow cytometric fluorescence origin did not provide coherent results. Another approach is to chemically force chlorophyll fluorescence with DCMU (Furuya and Li, 1992) for evaluating photosynthetic rates of natural populations. At very high photon densities such as in focused laser beams, 'exciton annihilation' or photo-bleaching may reduce fluorescence yield (Chekalyuk *et al*., 1992; Van den Engh and Farmer, 1992). Another question is whether the amount of absorbed quanta is (dis)proportional to the amount of intracellular pigment owing to the self shading effect, also called packaging effect (Duysens, 1956). At the small size range the packaging effect is not significant; Kerker *et al*. (1982) showed a linear relation between fluorescence and size of small calibration beads. Natural chlorophyll absorbs light more efficiently, and obviously phytoplankton cells may be much bigger. Sosik *et al*. (1989) showed that analyses of *Hymenomonas carterae* and *Amphidinium carteri* are influenced by the packaging effect. Calibration is required for accurate use of fluorescence as a size indicator (Legner, 1990). Flow cytometric 'pump and probe' analysis was demonstrated by Olson and Zettler (1995). The low laser power required to measure the constant fluorescence part, impaired the analysis of small cells. Better results were obtained with a so-called 'pump during probe' technique and using an infrared laser for particle detection to preserve the dark adapted state of the particles (Olson *et al*., 1996; Chekalyuk *et al*., 1996). The intensities used for pump and probe are orders of magnitude smaller than used in the studies mentioned above. The conclusion seems justified that more investigation of fluorescence rise times in the microsecond area, at a range of controlled flow cytometric illumination conditions, including estimation of packaging and annihilation effects, is required for a better understanding of these phenomena.

The total chlorophyll fluorescence, obtained by adding the individual particle fluorescence values, correlates well to spectrophotometrically analysed fluorescence per volume of sample (Hofstraat, 1991; Jonker, 1995). Especially for larger cells and colonies, these measurements require a dedicated optical measuring system like in the OPA as was shown for natural *Microcystis* colonies (Dubelaar and van der Reijden, 1995a). Based on this relationship the contributions of different groups of algae to 'biomass' can be assessed, which give more valuable information than counting of cells and colonies of different size alone. The combination of data on concentration, species and group discrimination and quantitatively measured chlorophyll fluorescence makes it possible to estimate the contribution of different species and groups to the total phytoplankton biomass. In vivo phytoplankton pigments have broad and overlapping excitation and emission characteristics, but the choice of laser wavelength and detection band influences the fluorescence measurement efficiency for a certain pigment. Fluorescence emission and excitation characteristics have been demonstrated as tools to classify groups such as cyanobacteria, cryptophytes, chlorophytes and prasinophytes, bacillariophytes and dinophytes based upon spectrally similar accessory pigments within these groups (Yentsch and Yentsch, 1979; SooHoo *et al*., 1986; Hilton *et al*., 1989; Hofstraat *et al*., 1990, 1991, 1994). Olson *et al*. (1989) simultaneously used Coulter-volume, intensity and polarisation of forward scatter, right-angle scatter, and fluorescence. From 26 laboratory cultures, the two cryptophytes and the rhodophyte, the coccolithophorids, and chlorophytes could be distinguished from others. Instead of fluorescing themselves, accessory (antenna) pigments rather increase chlorophyll-a fluorescence by energy transfer, the exception being phycocyanine and phycoerythrine

containing species. Therefore, excitation spectra probe the spectral properties of pigments capable of energy transfer to chlorophyll a (Owens, 1991). This could encourage the application of flow cytometers with three or more excitation beams. These instruments are the most complex, unfortunately, especially the electronic timing circuitry which correlates the data from one cell, coming from three lasers. Artificial neural net analysis (Boddy *et al*., 1994a; Wilkins *et al*., 1994, 1996) showed that 20-40 species can be sufficiently discriminated based on endogenous fluorescence and light scatter alone, but also showed that discrimination by this technique is not primarily based on taxonomic group identification.

Exogenous fluorescence

There are several options to extent analysis of phytoplankton by staining specific components of the cell. These fall apart in A: very specific but normally nonquantitative techniques in order to discriminate species and B: techniques for quantitative analysis of various constituents and physiological conditions of cells like DNA content. The dye can be fluorescent or the product of the dye and the cellular component of interest may be fluorescent. Except for the membrane binding dyes, the dyes have to enter the cell to interact with their goal substances. This can be used as a diagnostic technique for estimation of membrane integrity, otherwise the membranes have to be perforated to let the dye in (electroporation technique is described by Berglund and Starkey, 1991). An example is the use of propidium iodide for live/dead cell discrimination. Cytochemical stains are used to stain protein, DNA, RNA, lipids and membranes. Hull *et al*. (1982) presented staining techniques for nuclear DNA in algae. Schäfer *et al*. (1996) applied three-laser flow cytometry for simultaneous measurement of photosynthesis pigments and protein content using FITC of phytoplankton populations in lakes and rivers. Edvardsen and Vaulot (1996) used cell size and relative DNA content for ploidy analysis of Prymnesiophyceae spp. González-Gil *et al*. (1998) used flowcytometric measurement of ELF (enzyme-labeled fluorescence), a new insoluble fluorogenic substrate for alkaline phosphatase to probe the phosphorus (limitation) status of individual cells. The sensitivity of flow cytometers allows the detection of very small quantities of fluorescence, down to a level of a few thousand fluorescent molecules. This allowed the development and application of immunochemical labelling techniques. Double staining allows the assessment of more than one property at the same time, e.g. the double staining of bacteria with a DNA and a protein stain to monitor cell volume as a function of cell cycle (Steen *et al*., 1982). Zubkov *et al*. (1999) developed a flow cytometric assay for measuring protein content of marine planktonic bacterial cells using SYPRO staining. Advances in fluorescent probe technology offer realistic approaches for direct cell identification, viability assessment and responses to environmental changes using basic, single light-source flow cytometers (Porter *et al*., 1997). Jonker *et al*. (2000) gives an overview on the use of fluorescent probes in aquatic ecology.

Immunotechniques

An early overview on quantitative immunofluorescence in flow cytometry and related staining techniques was given by Visser *et al*. (1978). Ward and Perry (1980) presented an immunofluorescent assay for the marine ammonium-oxidizing bacterium *Nitrosococcus oceanus*. Clones of marine chroococcoid cyanobacteria were analysed by Campbell *et al*. (1983) using immunofluorescence. Antibodies to eukaryotic cells (to probe pigment types and/or cell wall composition) were presented by Shapiro *et al*. (1989) and Campbell *et al*. (1989). Antibodies were found to various cellular molecular constituents (Yentsch, 1981; Yentsch *et al*., 1988). Flow sorting gated on forward light scatter and FITC labelled anti-*Cryptosporidium* is being used in water quality analysis as a quantitative preconcentration method, which allows routinely screening of hundreds of litres of water for C*ryptosporidium* oocysts (Vesey, 1994). The antibody is not 100% specific, but the highly infectious oocysts are counted microscopically from the sorted fractions much quicker than before, without enrichment. 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, allow immunofluorescent detection in flow of the dinoflagellates *Prorocentrum micans, Gyrodinium aureolum* and *Gymnodynium nagasakiense*. Simon *et al*. (1997) identified the toxic algae *Chrysochromulina* and *Prymnesium* species using fluorescent or chemiluminescent oligonucleotide probes. For a review of immuno flow cytometry, see Peperzak *et al*., 2000).

DNA and RNA quantification

DNA can be fluorescently stained with many fluorochromes. Flow cytometric determination of phytoplankton DNA in cultures and field samples was reported by Yentsch *et al*. (1983b), Bonaly *et al*. (1987), and Boucher *et al*. (1991) from cultured samples stained with DAPI, which can be excited with UV light. The DAPI-DNA fluorescence was related to cell DNA content over almost 4 orders of magnitude. In natural populations, the fraction of particulate DNA contained in photosynthetic picoplankton could be computed. Currently, a new series of fluorochromes are being applied for high-resolution DNA quantification by flow cytometry. Among these are TOTO-1, SYBR-Green, Picogreen, SYTOX green (Marie *et al*., 1996; Li *et al*., 1995; Marie *et al*., 1997; Guindulain *et al*., 1997) and YOYO-1 (Jochem and Meyerdierks, 1999). They have major advantages over previously used dyes like DAPI and propidium iodide. They can be excited at 488 nm, the standard laser wavelength for most benchtop instruments, and result in green fluorescence which hardly interferes with pigment fluorescence (Chl-a), and are specific for double stranded DNA, instead of also staining RNA (Pan and Cembella, 1996; Pan *et al*., 1999). A comparison of seven blue nucleic acid SYBR and SYTO series dyes for flow cytometric enumeration of bacteria in aquatic systems by Lebaron *et al*. (1998a) showed that SYBR-II and SYTO-9 are the most appropriate dyes for bacterial enumeration in nonsaline waters and can be applied to both live and dead bacteria, whereas SYBR-II is more appropriate than SYTO dyes for seawater samples. SYTOX Green however underestimates the fraction of dead bacterial cells within starved populations (Lebaron *et al*., 1998b, see also Gasol and del Giorgio, 2000) and its application to natural samples should be considered with caution. Veldhuis *et al*. (1997a) established a nice data set of DNA content of individual phytoplankton species. They also showed that there is a good correlation between DNA content, as measured with these dyes, and phytoplankton biomass. This correlation is better than the correlation between biomass and Chl-a fluorescence which is influenced significantly by the light history of the cells. The next important step would be to transfer this to field samples. The ratio between RNA and DNA can be used to discriminate actively growing cells from resting cells. The application of e.g. SYTO 13 allows assessment of bacterial abundance (Del Giorgio *et al*., 1996; Gasol and del Giorgio, 2000); SYBR Green allows for discrimination between heterotrophic bacteria and autotrophic *Prochlorococcus* cells (Marie *et al*., 1997). Binder and Liu (1998) examined the relationship between growth rate and rRNA content in a marine *Synechococcus* strain by a combination of flow cytometry and whole-cell hybridization and found that both these methods correlated well in determination of total cellular RNA, which varied in a similar manner as growth rate, supporting the notion that measurements of cellular rRNA content might be useful for estimating in situ growth rates in natural *Synechococcus* populations. Brussaard *et al*. (1999) showed that flow cytometry can be a useful tool to discriminate between virus infected and noninfected phytoplankton cells by detection of de novo synthesis of viral DNA and possible digestion of host DNA.

Cell cycle analysis

Whereas the connection between cell division and the need for metabolites and photosynthates is obvious, the mechanism of light acting upon cellular DNA synthesis regulation is not so clear (Yee and Bartholomew, 1988). Flow cytometric DNA analysis is a helpful tool in this respect (Chisholm *et al*., 1986). Brzezinski *et al*. (1990) examined the role of silicon availability on cell-cycle progression in marine diatoms. Examination of DNA histograms allowed the localization of the effect of silicon deprivation in terms of progress through G1, S, and G2+M phase. Yee and Bartholomew (1988) studied light regulation of the cell cycle in *Euglena gracilis bacilaris*. Euglena grown under phototrophic conditions are easily synchronized to a 12 h light-12 h dark regime. By inoculating stationary phase, nondividing cells into fresh media and exposing the diluted cells to either light or darkness, it was observed that initiation of DNA synthesis for the cell division cycle is light dependent. Commitment to the cell cycle requires exposure to more than 6 h of light, supposedly to allow the accumulation of an initiating factor that will enable DNA synthesis to begin. Flow cytometry analysis showed that once cells are committed to the cell cycle, they complete the cycle in the dark, so mitosis is a light-independent step. Lefort *et al*. (1987) used DNA flow cytometry to study cell cycle blockade of vitamin B12-starved cells. Binder and Chisholm (1990) studied the relationship between DNA cycle and growth rate in

Synechococcus-sp strain PCC 6301. This cyanobacterium was shown to contain multiple chromosome copies even at very low growing rates. Evidence was found for asynchronous initiation of DNA replication. Vaulot *et al*. (1995) elegantly showed that DNA replication occurred in the afternoon by analyzing *Prochlorococcus* in samples from different depths using Hoechst 33342. The next step in *in situ* growth rate analysis is to combine specific detection of single species with cell cycle analysis. Specific detection can be done with *in situ* hybridization or with monoclonal antibodies. Peperzak *et al*. (1998) showed that flow cytometry can be used for the analysis of the dial DNA cycle.

Identification of species and groups using ribosomal RNA-targeted nucleic acid probes

The use of ribosomal RNA probes for flow cytometric identification of both individual species and taxonomic groups is very promising. Parts of the ribosomal RNA sequences were highly conserved during evolution and the differences in rRNA sequences correlate well with evolutionary relations. Fluorescently labelled rRNA probes are relatively small and penetrate easily in fixed cells (Amann *et al*., 1990). There they hybridize specifically to the target sequences. This is very useful for fluorescent *in situ* hybridization (FISH), a process which can be visualized using both microscopy and flow cytometry. Recently, the application of rRNA probes for detection of phytoplankton species and groups was established (Lim *et al*., 1993; Simon *et al*., 1995; Knauber, 1996; Rice *et al*., 1997; Partensky *et al*., 1997, Jonker *et al*., 2000). Lange *et al*. (1996) have shown that target regions specific for the class Prymnesiophyceae and the genus *Phaeocystis* (Hariot) Lagerheim could be identified from 18S rRNA coding regions, and two complementary probes were designed. Detection of whole cells hybridized with these probes labelled with fluorescein isothiocyanate (FITC) was difficult using epifluorescence microscopy because autofluorescence of the chlorophylls seriously interfered with the fluorescence of the probes. In contrast, flow cytometry proved very useful to detect and quantify the fluorescence of the hybridized cells. Hybridization conditions were optimized, especially with respect to formamide concentration. Both probes were tested on a large array of both target and non-target strains. Positive and negative controls were also analysed. Specificity was also tested by adding a competing non-labelled probe. Whereas probe PHAEO01 seems to have good specificity, probe PRYMN01 appeared less specific and must be used with stringent positive and negative controls. A large number of rRNA sequences has already been analysed and is available through internet. The use of a 18S rRNA probe for detection of *Cryptosporidium* was established by Vesey (1996). This allows for a very quantitative technique for analysis of *Cryptosporidium.*

Monoclonal antibodies and/or other molecular probes are used for aquatic bacteria and phytoplankton analysis by 6 of the 19 survey institutes; 4 other institutes say they will in the future.

Applications: *The survey institutes all carry out species or (pigment or taxonomic) group identification in field samples by flow cytometry, mostly taxonomic groups based on pigment analysis, such as prochlorophytes, cyanobacteria, (sub-types of) Synechococcus e.g. phycocyanin- and phycoerythrin-rich Synechococcus spp., cryptophytes, dinoflagellates and diatoms. Furthermore, eukaryotic picoplankton (three types of picoeukaryotic algae), and nanoplankton can be identified. Size is used by one institute as extra indicator to identify 6 groups in North Sea waters: Phaeocystis (only blooms), Mesodinium, Rhodomonas, Dinoflagellates and Diatoms <25 µm, 25-100, >100 µm. Gymnodinium mikimotoi is being identified using monoclonal antibodies. Heterotrophic bacteria are distinguished with DNA stains, while some molecular probes are also used. Applications mentioned included*

- Phytoplankton photophysiology, ecophysiology, toxicology, metabolic activity (3 times)
- The relationship between phytoplankton, bacteria and detritus (4 times), bacterial sorting to discriminate active cells (twice), size distribution of bacteria (2 times)
- Grazing impact studies (6 times), food selectivity of cockles
- Isolation of strains(e.g. gonyaulacoid cells) from natural populations by sorting (twice), isolation of diatoms transformed with GFP gene
- In situ hybridization with taxon-specific rRNA probes

Phytoplankton abundance

Basic characterisation of particles by flow cytometry can be done on a routine basis, such as a division in organic and inorganic particles, living and nonliving organic particles, e.g. Moreira-Turcq

and Martin (1998). Flow cytometry allows rapid phytoplankton counting and sizing based on the chlorophyll fluorescence, and limited identification of taxonomic groups and species (Li and Wood, 1988; Yentsch and Yentsch, 1979; Yentsch and Pomponi, 1986; Olson *et al*., 1989; Hofstraat *et al*., 1990, 1994; Blanchot and Rodier, 1996; Binder *et al*., 1996; Li, 1997; Veldhuis and Kraay, 1990; Wood *et al*., 1985). Clearly, pigment analysis using flow cytometry is much cruder than, for example, HPLC, but main pigments can be studied with success (Olson *et al*., 1990; Hess *et al*., 1996). Using the specific, immuno- and molecular probes enables the determination of intracellular substances such as lipid droplets in diatoms and toxins in dinoflagellates, and allows more specific species/group characterisation and discrimination (Bloodgood *et al*., 1987; Simon *et al*., 1994, 1995; Rao *et al*., 1991). Biodiversity of unicellular pico- and ultraplanktonic eukaryotes was studied by Chrétiennot-Dinet and Courties (1997). As very little is known about sexuality and life-cycles of these tiny algae, a whole set of complementary techniques including flow cytometry appears necessary to ensure a reliable assessment of identification and diversity. Gasol *et al*. (1999) estimated bacterial abundances with epifluorescence microscopy methods and with flow cytometry (SYTO13) and found good correspondence between microcosm experiments and coastal Mediterranean water. Flow cytometry is likely to prove useful for detecting the low-level occurrence of harmful species, giving early warning of the probability of bloom development. It enables monitoring of bacteria in seawater (Button and Robertson, 1989, 1990; Robertson and Button, 1989; Wiebenga *et al*., 1997; Zubkov *et al*., 1998). Flow cytometry allows higher sampling frequencies, important for the execution of ship transects, depth profiles and also incubation experiments to study the distribution and composition of phytoplankton populations, including diurnal variations (Tarran and Burkill, 1993; Burkill, 1987; Olson *et al*., 1985; Li, 1989; DuRand *et al*., 1994; Jacquet *et al*., 1998). Flow cytometry has also been used in all Joint Global Ocean Flux Studies (JGOFS) to date, particularly studies of the dynamics and distribution patterns of pico-phytoplankton, ultra-phytoplankton, and bacteria (Binder *et al*., 1996; Detmer and Bathmann, 1997), with diel cycling and biomass assessed from flow-cytometer cellular light scatter observations (Blanchot *et al*. 1997; Gin *et al*., 1999, respectively), the importance of *Prochlorococcus* to community structure (Campbell *et al*., 1994) and the effect of environmental forcing on microbial community structure (Campbell *et al*., 1998), investigations in the microbial food web structure in the Arabian Sea by Garrison *et al*. (2000), revealing a correlation between dominance of large phytoplankton, primarily diatoms, and the seasonal maxima of mass flux during the SW Monsoon. Landry *et al*. showed in 1996 that, while picoplankton account for most of the chlorophyll biomass and primary production in the central equatorial Pacific, their abundances and distributions are relatively stable and conservative while other populations, such as diatoms, respond more dramatically to environmental forcing. Other JGOFS missions included microzooplankton grazing in the central equatorial Pacific (Constantinou *et al*., 1995) and the Arabian Sea (Reckermann and Veldhuis, 1997), and flow cytometry based studies of energetics and growth kinetics of a deep *Prochlorococcus* spp. population in the Arabian Sea (Johnson *et al*., 1999), populating almost exclusively the detected secondary fluorescence maxima (SFM).

Ecology and physiology

Flow cytometers can play an important role in the experimental verification of ecological models (Campbell *et al*., 1997). These models include spatial and/or temporal distributions of species and groups (Olson *et al*., 1990; Partensky *et al*., 1996; Shimada *et al*., 1993, 1995; Tarran *et al*., 1999; Vaquer *et al*., 1996; Vaulot *et al*., 1990; Zubkov *et al*., 1998, 2000), size distributions and population dynamics, but can also consider flow cytometrically probed status of life cycle (Binder and Chisholm, 1995; Brzezinski *et al*., 1990; Van Bleijswijk *et al*., 1994; Van Bleijswijk and Veldhuis, 1995; Green *et al*., 1996; Vaulot and Marie, 1999) allowing growth rate determination (Veldhuis *et al*., 1997b), and physiological properties of the cells such as metabolic activity (Dorsey *et al*., 1989; Jellet *et al*., 1996; Del Giorgio *et al*.,1997). Shalapyonok *et al*. (1998) reported ultradian growth (faster than 1division per day) observed for the widespread marine prokaryote *Prochlorococcus*, even though cell division was strictly phased to the light-dark cycle. Correlation of metabolic with flow cytometric characteristics is a feasible means of investigating the heterogeneity of phytoplankton metabolic state in the marine environment. The response to changes in light conditions (Armbrust *et al*., 1989, 1990; Gerath and

Chisholm, 1989; Vaulot *et al*., 1986), nutrient availability (Trousselier *et al*., 1997; Vaulot *et al*., 1987; Veldhuis and Kraay, 1993; Graziano *et al*., 1996; Zettler *et al*., 1996; Timmermans *et al*., 1998; Lebaron *et al*., 1999) and kinetics (Button and Robertson, 2000) can be assessed. Lipschultz (1995) measured nitrogen-specific uptake rates of marine phytoplankton by flow cytometry. Huisman *et al*. (1999) elegantly used flow cytometry in competition experiments in order to validate models describing competition for light using fresh water species from eutrophicated systems. Other applications include the investigation of microbial food webs (Cowles *et al*., 1988; Cucci *et al*., 1985, 1989; Gerritsen and Sanders, 1987; Lesser *et al*., 1991; Sellner *et al*., 1994; Hansen *et al*., 1996; Reckermann and Veldhuis, 1997) and the evaluation of the effect of toxic substances on algae (Gala and Giesy, 1990, 1994), or toxic algae on zooplankton (Turner *et al*., 1998). Boelen *et al*. (2000) used flow cytometry for phytoplankton species composition and size analysis in a study of ultraviolet-B (UVB) radiation induced DNA damage in picoplankton assemblages in the tropical Atlantic Ocean, indicating that radiation damage is size dependent, and mean received doses are reduced by wind-induced mixing. Mostajir *et al*. (1999) found retardation of cell division, inhibition of photosynthesis and cell size enlargement of Prymnesiophyceae provoked by UVB. Waite *et al*. (1995) measured sugar-containing compounds on cell surfaces of marine diatoms with flow cytometry using concanavalin A. Measurements of effects of iron stress on chromatic adaptation by natural phytoplankton communities in the Southern Ocean by Van Leeuwe *et al*. (1998) showed that iron did affect the pigment composition, but the efficiency of electron transfer. Reed *et al*. (1999) followed changes in the neutral lipid content of actively swimming zoospores of the palm kelp *Pterygophora californica* by flow cytometry using the fluorescent stain Nile Red. A flow cytometric approach to assess the environmental and physiological status of phytoplankton was presented by Demers *et al*. (1989). The analysis of natural phytoplankton populations can indicate changes in water quality and environmental stress (Olson and Chisholm, 1986; Olson *et al*., 1986; Parpais *et al*., 1996), starvation conditions (Joux *et al*., 1997), and contamination by anthropogenic inputs (Berglund and Eversman, 1988; Bonaly-Cantarel, 1988; Firth *et al*., 1994; Cid *et al*., 1996, 1997; Cavender-Bares, 1999; Faber *et al*., 1998; Franqueira *et al*., 1999).

Size and biomass

Many biological processes involved in biogeochemical fluxes, e.g. production, respiration, grazing, sinking, aggregation, are linked to the biomass and size distributions of bacteria (Button and Robertson, 1989, Robertson *et al*., 1998) and phytoplankton (Rodriguez and Li, 1994, Gin *et al*., 1999). For that reason the development of flow cytometric techniques for particle size determination was advocated by Legendre and Le Fevre (1991). Shipboard based instruments allow fast assessment of abundance and biomass variations (Burkill, 1987; Borsheim *et al*., 1989; Zohary *et al*., 1998; Zubkov *et al*., 1998, 2000), to probe three dimensional patchiness dynamics such as in eddies (Tintore *et al*., 1998). Buck *et al*. (1996) estimated living carbon biomass of microplankton, nanoplankton and picoplankton using a combination of dual beam flow cytometry and epifluorescence microscopy. The contribution of picophytoplankton to biomass, productivity and community structure was the subject of various studies using flow cytometry (Li, 1994, 1995; Charpy and Blanchot, 1998, 1999; Liu *et al*., 1997; Gasol *et al*, 1997, 1999). Pichard *et al*. (1997) used flow cytometry to show the utility of group-specific gene probes for examining the relation of active phytoplankton community structure to the fixation of inorganic carbon. Weisse and Kirchhoff (1997) measured feeding rates and food selectivity of the heterotrophic freshwater dinoflagellate *Peridiniopsis berolinense* by flow cytometry and other techniques. Bougrier *et al*. (1997) used flow cytometry to study preingestive selection of different microalgal mixtures in *Crassostrea gigas* and *Mytilus edulis*. A rapid cytometric method assessing the distribution of ingested bacteria in nanoflagellates was presented by Bratvold *et al*. (2000), investigating grazing behaviour of cultures of *Rhynchomonas nasuta* and *Paraphysomonas vestita*. Their results suggest a heterogeneous population composed of subgroups of flagellates with different grazing rates, although the specific biological implications of the statistical models used with regard to the number of flagellate subgroups remain to be proven. Grazing was also studied by Christaki *et al*. (1999) who found that *Prochlorococcus* may be less subject to ciliate predation than *Synechococcus*, and Kenter *et al*. (1996) who recorded the feeding of *Balanion planctonicum* on the cryptomonad *Rhodomonas* sp. in vivo at 2-3 min intervals by flow cytometry. Vazquez-Dominguez *et al*. (1999) pre-

sented some succesful flow cytometric experiments to estimate grazing rates on bacteria with fluorescently labeled tracers, and Kuipers and Witte (1999) demonstrated the use of flow cytometry to obtain significant results in a study of the impact of microzooplankton community grazing on different size classes of algae. Pile *et al*. (1997) studied trophic effects of sponge feeding on four types of picoplankton, and Monger *et al*. (1999) examined the influence of cell-surface hydrophobicity. Gin *et al*. (1999) studied seasonal and depth variation in microbial size spectra at the Bermuda Atlantic time series station, using dual-beam flow cytometry to generate concentration and biomass size spectra of bacteria and phytoplankton.

Almost half (8) of the survey participants carry out quantification of growth/production rates (basing on DNA / cell cycle analysis) of phyto-/bacterioplankton.

The ocean colour is a prime source of information (Morel and Prieur, 1977). Remote sensing data may be utilized in calibration and validation of hydrodynamic and ecological models, and for instance the assessment of the spatial variability of biomass within a bloom area, or on a larger scale the photosynthetic carbon fixation in the world oceans. Data interpretation depends on atmospheric correction methods and bio-optical algorithms, calibrated with locally (on site) acquired entities such as extracted chlorophyll. Calibration with 'sea truth' data on the particle level is complicated (Lewis and Cullen, 1991) but feasible, for instance for coccolithophore blooms (Holligan and Balch, 1991; Balch *et al*., 1992, 1993), or a bio-optical classification of sea particles (Ackleson and Robins, 1990). Although flow cytometers can measure cellular light absorption indirectly only (Perry and Porter, 1989), other entities such as fluorescence, forward and perpendicular light scatter are useful to assess particle optical properties and the relationship with the *in situ* light fields (Spinrad, 1984; Ackleson *et al*., 1993; Cullen *et al*., 1988; DuRand and Olson, 1996).

The general idea is, although it is obviously not a trivial matter, that flow cytometry data could be related to measurements of bulk light scatter or attenuation data measured with other instruments. Some scientific work and projects are being executed in this field currently. Some institutes compare flow cytometry data with microscopic counts and optical telemetry (shipboard SpectraScan) data from specific stations. The main advantage of the flow cytometer is the straightforward evaluation of phytoplanktonic biomass within main groups. *Another aspect is that major changes in ocean colour are based on new production (hence large) species. If cytometers were to be technically tuned towards larger organisms (probably even aggregates) and larger measuring volumes they could be useful for that purpose, in addition to the importance of flow cytometry for characterising small particles.*

DISCUSSION

A high potential of the technique was acknowledged by all participants of the survey. It was stated that the direct on-line estimation of phytoplankton biomass and the ability to discriminate between different phytoplankton groups allows analysis of undisturbed natural samples (growth, grazing, the spatial or seasonal evolution of populations). Also, the application of specific fluorescent stains (bound to antibodies, or as DNA probes) may provide early detection of plankton groups of special interest, e.g. toxic species. By combining auto- and artificially fluorescent properties, potential developments arise include monitoring, in situ growth rates, biomass estimation of both autotrophs and heterotrophs, ecosystem processes, physiological status of microbial assemblages such as nutritional status, viability, photosynthesis, and various stress physiology. On a broader scale, we can use a similar approach to study primary productivity, and new production in the field. Flow cytometry also carries potential for the determination of small particles such as bacteria. Besides limitations as mentioned previously, an inherent drawback of flow cytometry is that it can only differentiate particles based on their optical characteristic as seen by the PMT's. This is better than the eye for the very small cells (picoplankton), and vice versa for the larger cells. The species resolution of field samples done by flow-cytometry is far below the Utermoehl-microscopy. The expectation that this situation can improve if the lack of specific fluorescent probes is reduced was widely supported in the survey. The laborious work to establish them remains a hurdle, as well as perhaps the human factor: ecologists prefer microscopic monitoring data. With regard to reproducibility it was mentioned that there is a real need for (i) standardization of flow cytometric analysis of field samples,

and (ii) continuous checking against other independent methods (such as microscopy).

Clearly, applications of flow cytometry in the field of aquatic sciences are numerous and expanding. It is not possible to define general requirements: shipboard analysis requires robust, easily transportable equipment; oceanography requires high sensitivity analysis of small cells; coastal and fresh water applications require a large dynamic range. Many physiological experiments and species identification using molecular techniques can be done with one or two parameters, whereas species identification based on inherent optical properties requires large numbers of independent entities measured from a single particle. Broadly used dedicated commercial equipment is not expected soon, but the general instrumental developments are promising. Data acquisition and processing gets more efficient, lasers and detectors get smaller, PC's get faster and hard discs get bigger, costs decrease. Instrument size, -operation, -costs and -reliability and data handling will not remain bottlenecks for long. Developments over a longer period will allow real-time in-situ measurements. These developments facilitated the development of the buoy based flow cytometer CytoBuoy with radiotransmission (viz. Fig. 1) to shore (Dubelaar *et al.*, 1999; Dubelaar and Gerritzen, 2000). A submersible version will also be deployed on missions with an autonomous underwater vehicle (AUV), while Olson and Sosik (1999) developed a submersible flow cytometer without a sheath fluid system. Much effort has to be devoted to standardization of sampling and preservation and of reporting and data analysis. This also requires intercalibration of flow cytometers. Also the systematic investigation of the phenomena governing the light scatter and fluorescence of phytoplankton cells as measured flow cytometrically would contribute to a better understanding of these analysis tools. This may facilitate the intercalibration between flow cytometers and possibly the intercalibration of data from flow cytometers with remote sensing data and other 'bulk' techniques (Dubelaar *et al.*, 1994; Geider *et al.*, 1998). It would also support the further development and use of 'pump/probe' capabilities in flow cytometry to measure photosynthetic parameters in a single cell. If analysis of major functional groups within the phytoplankton and bacteria becomes routine, it will be possible to base time-series on flow cytometric data, with the advantages of higher frequencies in time and space.

ACKNOWLEDGEMENTS

We thank the editors of this issue for encouraging us to write this paper, and we thank the reviewers for their valuable contribution. For responding to the survey we gratefully thank Dr. P.H. Burkill (Plymouth Marine Laboratory, United Kingdom), Dr. Raffaella Casotti (Stazione Zoologica 'A. Dohrn', Italy), Dr. Kent Cavender-Bares (Dept. of Biology, Massachusetts Inst. of Techn. USA), Dr. Claude Courties (Laboratoire d'Hydrobiologie Marine, Université Montpellier II, France), Dr. Alex Cunningham (Univ. of Strathclyde, Dept. of Physics and Appl. Physics, Scotland (UK)), Dr. Michel Denis (Centre d'Oceanologie de Marseille, France), Dr. Ken Furuya (Department of Aquatic Bioscience, University of Tokyo, Japan), Dr. Josep M. Gasol (Institut de Ciencies del Mar, Catalonia (Spain)), Dr. Hans Hoogveld (Netherlands Institute of Ecology, Centre for Limnology, The Netherlands), Dr. Frank J. Jochem (The University of Texas at Austin, USA) Dr. Ulrich Kenter (University of Konstanz, Dept. of Limnology, Germany), Dr. Philippe Lebaron (Université Pierre et Marie Curie, Observatoire Océanologique, France), Dr. William Li (Bedford Insitute of Oceanography, Canada), Dr. Youlian Pan (Inst. for Marine Biosc., National Res. Council of Canada, Canada), Dr. Falk Pollehne (Institut für Ostseekunde, Warnemünde, Germany), Dr. Marcus Reckermann (Univ. Kiel, Research and Technology Center Westcoast, Germany), Dr. Jaime Rodríguez (Dpto. de Ecologia, Fac. de Ciencias, Universidad de Malaga, Spain), Thomas Rutten MSc (National Institute for Coastal and Marine Management, The Netherlands), Dr. Marcel Veldhuis (Netherlands Institute of Sea Research, The Netherlands).

Information was used from the internet sites of Station Biologique CNRS - INSU - UPMC (www.sb-roscoff.fr) Roscoff, France; J.J. MacIsaac Facility for Indiv. Particle Anal. (www.bigelow.org); West Boothbay Harbor, Maine, USA; Institut für Meereskunde Kiel (www.ifm.uni-kiel.de) Kiel, Germany.

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