SCI. MAR., 64 (2): 183-195 SCIENTIA MARINA 2000

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

Probing the physiological state of phytoplankton at the single-cell level*

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SUMMARY: Probing the physiological state of phytoplankton at the single-cell level provides valuable insight in ecological studies as well as in environmental monitoring of pollution or UV impacts. This paper reviews the recent progress in assessing the physiological state of phytoplankton with flow cytometry by inherent cell properties such as cell size and chlorophyll autofluorescence, specific fluorescent dyes, and newly developed molecular probes and enzyme substrates. It is reported how nitrogen and iron limitation as well as the effect of copper pollution could be derived from changes in cell inherent properties. Effects of Cu were also recorded by monitoring cell membrane potentials and esterase activity. Photosynthetic capacity of algae was assessed by changes in chlorophyll fluorescence with the electron transport inhibitor DCMU, by a cytometric adaptation of the pump-and-probe approach, and molecular probes for Rubisco. Antibodies were also applied to mark non-terminal stages in the cell DNA replication cycle, to detect non-proliferating cells, to assess DNA damage caused by UV-B radiation and to quantify diatom stickiness. Fluorescein diacetate proved useful to discriminate metabolically active from inactive cells and to reveal strategies of dark survival in algae. The activity of alkaline phosphatase was recorded by a new fluorigenic substrate ELF, and polyclonal antibodies against nitrate reductase (NR) provided measurements of the NR abundance. An outlook will show how recent developments in molecular probes might affect the future analysis of marine ecosystems and their communities.

Key words: flow cytometry, phytoplankton, physiology, molecular probes, fluorescent dyes.

INTRODUCTION

In times of increasing public awareness and sensitivity towards the environmental status of our oceans, reliable and conclusive biological monitoring becomes a politically as well as scientifically vital task. Changes in environmental conditions are often most easily and rapidly recognized by the reaction of unicellular organisms. Due to short generation times and a comparably simple organismic organization protists can react faster than higher organisms with high organizational complexity. Furthermore, their simple organization makes the detection of specific physiological reactions and conditions more feasible. Particularly the marine phytoplankton can serve as a valuable marker for environmental monitoring.

Environmental impacts and water pollution can affect organisms – and thereby the whole ecosystem and its functioning– although the water looks "chemically clean". To be able to detect such contamination based on physiological "properties" of the organisms will help to identify chronic environmental pollution and to provide early warning at sublethal pollution levels.

*Received May 4, 1999. Accepted January 18, 2000.

Besides these more applied aspects a variety of questions on how aquatic systems function still remain to be resolved. Among them, basic ones such as the persistence of High Nutrient – Low Chlorophyll areas (HNLCs) where phytoplankton is unable to proliferate and deplete high ambient nutrient stocks of nitrate and potential iron limitation is discussed (Martin and Fitzwater, 1988; Martin *et al.*, 1990), the control of nutrient uptake and regeneration kinetics in different pelagic communities, and the reaction of inter-species competition and food web dynamics on environmental conditions. More peculiar topics comprise, for example, the observation of algal accumulations in aphotic depths (Detmer *et al.*, 1993; Murphy and Cowles, 1997) and how or if they survive, or the specific conditions that allow harmful algal blooms to develop. Altogether, there are many occasions when insight into the physiology of phytoplankton is desirable within studies of aquatic ecosystems.

The uniqueness of flow cytometry lies in the ability to perform quantitative measurements of individual cells in large numbers and appreciably short time. Application to field samples might reveal different types of physiological responses of distinguished sub-populations of the phytoplankton community not accessible by bulk measurements and may yield insight into the competitive advantage or adaptation of single algal species/groups upon specific environmental conditions.

Flow cytometry can, therefore, take advantage of directly measurable, inherent cell properties or make use of fluorescent markers for cell components or metabolic processes. New insights into community dynamics such as taxonomy, specific activity or limiting factors are nowadays possible through the development of new optical and molecular probes of individual cell physiology: quantification of cell death (Veldhuis and Kraay, 2000), reproduction (Jochem and Meyerdierks, 1999; Veldhuis and Kraay, 2000), enzyme activity, nutrient status and others.

Among fluorescent markers, static and dynamic measurements can be distinguished: static measurements refer to cell components such as DNA, proteins and chemicals within the cell or on the cell surface whose staining, once achieved, remains stable. In kinetic measurements, the staining result or fluorescence yield depends on the time of analysis and relies – in contrast to static measurements – on live cells; fluorigenic enzyme substrates that evolve fluorescence upon the activity of the studied enzyme are common examples.

The scope of this contribution is to review the present state of physiological probes available to the cytometric analysis of phytoplankton.

INHERENT CELL PROPERTIES

When flow cytometry was initially adopted from medical research to marine studies (Yentsch *et al.*, 1983), the primary goal was the fast and reliable quantification of the newly discovered picophytoplankton, algal cells of less than 2 μm in size. So far, these organisms, whose ubiquity and importance not only in oligotrophic oceans (Stockner and Antia, 1986) but also in temperate and coastal areas (Jochem, 1988, 1989) was rapidly acknowledged, were accessible only by timely microscopic analysis (Hobbie *et al.*, 1977). Later, the advantage of flow cytometry providing cellular properties such as cell size and chlorophyll content for each individual cell measured without additional effort have also been employed for ecophysiological insights.

Cell size is derived from light scatter at low angles relative to the excitation light, commonly called Forward Angle Light Scatter (FSC). Although the refractive index, which is a function of the chemical composition, and the shape of cells might interfere with FSC measurements, FSC is commonly accepted as a relative measure of cell size, which increases linearly with the square of the cell diameter or cross section (Steen, 1991; Cunningham and Buonaccorsi 1992). Perpendicular or side-angle light scatter (SSC) reflects particle morphology and structure (Dubelaar *et al.*, 1987; Ackelson *et al.*, 1988). For small, picoplanktonic phytoplankton, however, SSC often provides a better measure of relative cell size because of higher sensitivity to small changes as compared to FSC. Minor differences in cell shape and morphology prevent the dependence of SSC on cell shape to bias its use as cell size proxy within this group of coccoid to ellipsoid organisms.

Chlorophyll autofluorescence, measured as red fluorescence >610 nm or >650 nm, depending on the individual instrument setup, has proven to be linearly related to cellular chlorophyll concentrations in laser-based instruments (Li *et al.*, 1993; Graziano *et al.*, 1996; Veldhuis and Kraay, 2000). Changes in cellular chlorophyll fluorescence, often considered as a sign of photoadaptation to decreasing light with increasing depth in the ocean (Veldhuis and Kraay, 2000), and changes of cell size have also been used

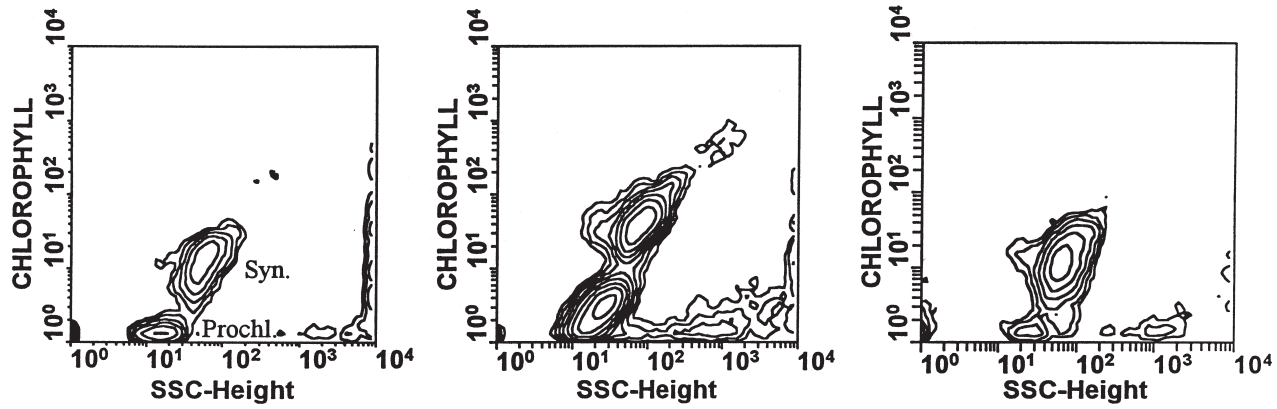

FIG. 1. – Effect of iron addition on picophytoplankton in the tropical North Atlantic, Bahamas: 2-parameter histograms of cell size (Side-angle light scatter, rel. units) vs. chlorophyll autofluorescence (rel. units). Field samples were incubated in quartz bottles in a cooled deck incubator with and without the addition of Fe (5 μM) in nutrient replete (f/20) conditions. (a) initial sample; (b) with Fe after 3 days; (c) without Fe after 3 days. Prochl. = *Prochlorococcus* sp.; Syn. = *Synechococcus* sp. Gelpke and Jochem, unpublished data.

to depict the physiological state of algae in nutrient or contaminant studies (Abalde *et al.*, 1995; Cid *et al.*, 1997).

Tightly coupled food web interactions often make it difficult to assess the potential limitation of a specific nutrient in addition experiments. In the North Atlantic, nitrate addition did result in higher cell abundance of *Prochlorococcus* sp. but not of *Synechococcus* sp.; increased cellular chlorophyll in both species, however, revealed nitrogen limitation in the latter as well (Graziano *et al.*, 1996). Similarly, iron enrichment in the Equatorial Pacific did not result in higher abundance of phytoplankton except for pennate diatoms; all phytoplankton showed, however, increased pigment fluorescence upon iron addition (Zettler *et al.*, 1996). In the tropical North Atlantic, cytometric assessment of cellular pigmentation also proved iron limitation of phytoplankton independent of the necessity to determine changes in actual growth rates (Fig. 1). Higher growth rates of phytoplankton due to nutrient additions might, thus, easily be masked by instantaneously higher grazing rates as well but physiological changes preserved within the single cells can be evidenced by flow cytometry.

An increase in cell volume upon copper pollution as previously reported from microscopic studies (Stauber and Florence, 1987) was cytometrically documented as an increase in FSC in *Dunaliella tertiolecta* (Abalde *et al.*, 1985) and *Phaeodactylum tricornutum* (Cid *et al.*, 1997). Heavy metals change the cell membrane permeability to small cations (Overnell, 1975), which may lead to the observed increase in cell volume (Cid *et al.*, 1997). At the same time, copper induced a significant increase in chlorophyll fluorescence in *P. tricornutum* (Cid *et al.*, 1996b). The inhibition of the electron flow in the photosystem II (PS2) reaction center at the donor site provokes a decrease in chlorophyll fluorescence, but inhibition at the acceptor site of PS2 will increase fluorescence (Samson *et al.*, 1988). Cytometric records of cellular chlorophyll fluorescence increase upon copper contamination evidence that copper acts on the oxidative side of PS2 (Cid *et al.*, 1996b). Whereas most toxicity tests on microalgae work on 96 hrs incubation periods, the reported changes in *P. tricornutum* could be documented by flow cytometry within 24 hrs after copper exposure (Cid *et al.*, 1997).

In mercury-lamp equipped cytometers with low excitation light intensity, linearity of live chlorophyll fluorescence to chlorophyll concentration is lost. Instead, on these instruments, DCMU-enhanced chlorophyll fluorescence can be used to assess the photosynthetic capacity of algal cells (Furuya and Li, 1992) as has been previously applied to bulk measurements of phytoplankton (Samuelsson and Öquist, 1977). DCMU (3-(3,4-dichlorphenyl)-1,1-dimethylurea) blocks the electron transport at the electron acceptor Q in PS2 and chlorophyll fluorescence is thereby maximized. The fluorescence enhancement by DCMU as compared to live fluorescence provides a measure for photosynthetic capacity. A FACS Analyzer with a mercury cadmium arc lamp provided a significant correlation between fluorescence enhancement and photosynthetic capacity as derived from $^{14}CO_2$-incubations (Furuya and Li, 1992).

More recently, the photosynthetic apparatus of phytoplankton has been monitored by saturating-flash or "pump-and-probe" fluorescence measurements (Falkowski *et al*., 1991), which uses a similar principle as the DCMU approach. The fluorescence of dark-adapted cells is measured by weak "probe" flashes of chlorophyll exciting light both before (F_0) and after (F_m) a saturating "pump" flash. The pump flash causes all the photosystem II (PS2) reaction centers to pass an electron to the primary electron receptor, thereby closing the PS2 to new excitement; excitations at such a reaction center have a higher probability of being re-emitted as fluorescence. For a cell with functional reaction centers, fluorescence from the second flash will be higher than from the first, and the difference (variable fluorescence, $F_v = F_m-F_0$) is related to the fraction of functional reaction centers (Olson and Zettler, 1995). F_v/F_m, which may range from zero to 0.65 (Falkowski *et al*., 1992), then represents a measure of PS2 efficiency.

Application of the "pump-and-probe" technique to oceanographic research demonstrated its usefulness to distinguish nutrient from light limitation in phytoplankton (Kolber *et al*., 1990, 1994; Geider *et al*., 1993; Kolber and Falkowski, 1993). However, since these applications are bulk measurements, they represent weighted average properties of all the fluorescent particles present in the sample. For example, an increasing proportion of detrital chlorophyll and an increasing degree in nitrogen limitation would both appear as a decrease in F_v/F_m (Olson *et al*., 1996). Flow cytometric analyses would not only prevent such confusion of different mechanisms influencing fluorescence results but would also provide insight into the natural variability of photosynthetic capacities among different species and among individuals of the same species in a given phytoplankton assemblage.

The potential for flow cytometric measurement of photochemical energy conversion efficiency of PS2 was demonstrated with a pump-and-probe approach by Olson and Zettler (1995). The results suggested, however, that this approach is limited by sensitivity problems associated with the low excitation intensity required for probing. Instrumental improvements comprised the addition of an infrared laser to trigger particle detection, whose wavelength does not interfere with PS2 activity, splitting the 488 nm laser into a high pump and low probe beam, and elongating the 488 nm laser spot for extended fluorescence measurement during passage time of the cells. Eventually, these instrumental modifications improved sensitivity and lead to a "pump-during-probe" approach. Instead of direct measurements of F_0 and F_m, fluorescence emission from each single particle is recorded over time scales of 100-250 μs during its passage through the elongated 488 nm laser spot (Olson *et al*., 1996, 1999). This system demonstrated that photosynthesis of pennate diatoms in the Southern Ocean was iron limited but that of cryptophytes was not (Olson *et al*., 1999). Still, this system is limited by sensitivity to the analysis of cells >2 μm (Olson *et al*., 1999), which would prevent its application in picoplankton dominated warm ocean regions. Time-resolved photon counting, however, provided reliable measurements on the photosynthetic system of the smallest known phytoplankton, *Prochlorococcus marinus* (Olson *et al*., 1996).

FLUORESCENT DYES FOR CELL CONSTITUENTS: STATIC MEASUREMENTS I

One of the most prominent and periodically changing constituents of living cells is the set of nuclear DNA, which undergoes replication and eventually doubling prior to cell division. A variety of specific dyes for double-stranded DNA have been successfully applied in flow cytometry to assess cell cycle stages and to derive in-situ growth rates (Jochem and Meyerdierks, 1999). For a detailed description of present achievements in cytometric cell cycle analysis, the reader is referred to Veldhuis and Kraay (2000) in this volume.

Cytometric application of $DiOC_6$ (3,3-dihexyloxacarbocyanine) and rhodamine-123 revealed changes in cell membrane and mitochondrial membrane potentials upon copper exposure in the marine diatom *Phaeodactylum tricornutum* (Cid *et al*., 1995, 1996a). Both membrane potentials increased with copper concentration, detectable 96 hrs after Cu exposure. Copper concentrations >0.1 mg l^{-1} induced degenerative processes in *P. tricornutum* related to disorders in membrane systems (Cid *et al*., 1996a) and 10 mg Cu l^{-1} provoked an acute increase in intracellular pH as evidenced from cytometric measurement of DCF (2,7-dichlorofluorescein; Cid *et al*. 1995).

The effects of mercury and cadmium on the intracellular level of nonproteinaceous thiols in the unicellular green alga *Tetraselmis tetrathele* were monitored by the fluorescent dye 5-chloromethylfluorescein (Satoh *et al*., 1999). Fluorescence inten-

sity within the cells was reduced by exposure to >3 μM $HgCl_2$ but not by exposure to $CdCl_2$ at concentrations of up to 1.0 mM. Depletion of nonproteinaceous thiols began within 30 min after mercury exposure, and cells were completely deprived of thiols after 2 hrs.

Other specific dyes for cell constituents such as Calcofluor White, which stains cellulose fibers and is used in dinoflagellate taxonomy (e.g. Fritz and Triemer, 1985; Jensen and Moestrup, 1997), or lipid stains such as Neutral Red and Nile Red were hitherto not exploited in flow cytometry although common in histochemistry and fluorescence microscopy.

MOLECULAR PROBES FOR CELL CONSTITUENTS: STATIC MEASUREMENTS II

The majority of molecular probes for static measurements of cell constituents and physiological status relies on the visualization of antigen-antibody reactions. The basic principle is the binding of the specific antibodies to the target of interest and subsequent visualization. The result of static measurements is, therefore, dependent on the concentration of the target antigen within the cell and the efficiency of antibody binding and antibody visualization.

Visualization can be achieved by using either fluorescently labelled antibodies, i.e. the antibody is bound to a fluorescent dye such as FITC, or by the reaction of a secondary antibody against immunoglobins of the host to the first antibody that is fluorescently labelled. In the latter case, the primary antibody is often conjugated to biotin and a fluorochrome-bound avidin acts as the secondary antibody. A more detailed technical overlook is provided by Peperzak *et al*. (2000).

Direct conjugates are useful when high concentrations of the target antigen are present or when cytometric multicolor analysis is planned with more than one antibody-dye-conjugate exhibiting different emission spectra. When high sensitivity is crucial, visualization by a secondary antibody is recommended because of higher fluorescence yield (Daley 1988): This happens because with direct conjugates only one antibody can react with the specific antigen site, whereas several secondary antibodies can react with the primary antibody. The detection signal is thereby increased manifold. Both approaches have been used in flow cytometry.

The commercially available antibody PCNA (anti-proliferating cell nuclear antigen-fluorescein, Boehringer Mannheim, Germany) successfully enabled estimates of growth rates in cultures of *Dunaliella tertiolecta* (Lin *et al*., 1995) and field populations of the marine diatom *Ethmodiscus rex* (Lin and Carpenter, 1995). PCNA is an auxiliary protein of polymerase-delta and therefore essential for cellular DNA synthesis; synthesis and abundance of PCNA are cell-cycle-dependent, both increasing markedly during S phase (Lin *et al*., 1994), the cell cycle phase of DNA replication. The commercial antibody cross-reacted with several marine phytoplankton species (*Skeletonema costatum*, *Thalassiosira weissflogii*, *Isochrysis galbana, Dunaliella tertiolecta*; Lin *et al*., 1994). Although not yet applied to flow cytometry results from fluorescence microscopy have been promising.

Statin, a 57 kDa nuclear protein, was recognized as a unique marker of quiescent cells, often also named G_0 phase cells in the DNA cell cycle. Using flow cytometry, monoclonal antibodies against statin could discriminate resting (G_0) from potentially proliferating (G_1) human fibroblasts within a cell fraction having the same DNA content (Pellicciari *et al*., 1995). The expression of statin was inversely correlated with that of PCNA. Although not tested in non-mammalian cells, statin might prove useful to detect resting or dormant cells among morphologically identical phytoplankton, provided statin is as conservatively expressed among different types of organisms as PCNA (Lin *et al*., 1994) seems to be.

Cytometric immunofluorescent detection of monoclonal antibodies against thymine dimers, first developed for medical research (Roza *et al*., 1988), illustrated the dynamics of DNA damage in marine phytoplankton upon UV-B radiation (Buma *et al*., 1995). Thymine dimers are frequently built upon DNA lesions under UV radiation (Tyrrell 1986), blocking the action of DNA polymerase (Setlow *et al*., 1963) and preventing genome replication. The cyclobutane ring of the dimers is broken by a group of enzymes known as photolyases, and this repair mechanism is induced by light with wavelengths of 330 to 450 nm (Buma *et al*., 1995). Thymine dimer-specific fluorescence in the marine diatom *Cyclotella* sp. was linearly related to the UV-B dose and no difference was detected in the vulnerability to UV-induced DNA damage between G_1-phase (non-replicating) and G_2-phase (replicating) cells (Buma *et al*., 1995). The specific growth rates of *Cyclotella* sp. (Buma *et al*., 1997) and *Emiliania huxleyi* (Gieskes and Buma, 1997) were inversely correlated with

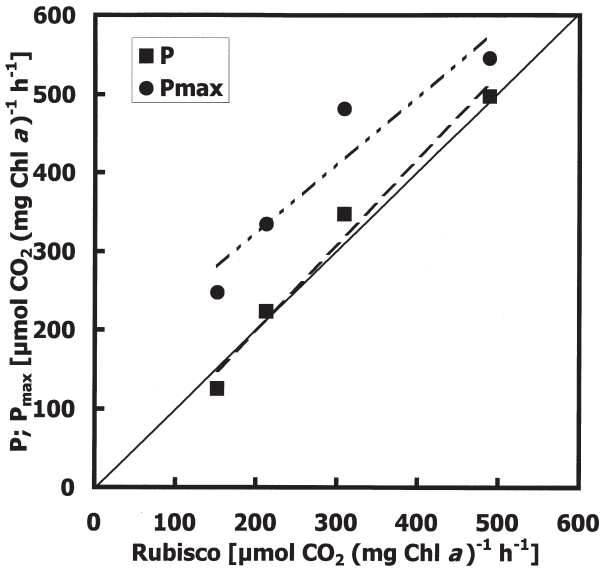

FIG. 2. – Relationship between ambient primary production (P) and maximum primary production (P_{max}) to maximum Rubisco activity (all in μmol CO_2 mg Chl.a^{-1} l^{-1}) in the chlorophyte *Scenedesmus ecornis*. Redrawn from table 1 in Mouget *et al.* (1993).

thymine dimer content in the cells. After UV-B exposure, thymine dimers were completely removed within 8-24 hrs under non-UV light (Buma *et al.*, 1996; Gieskes and Buma, 1997).

The search for non-radioactive techniques to assess primary production resulted in the development of antibodies against ribulose-1,5-bisphosphate carboxylase (Rubisco; Orellana *et al.*, 1988; Orellana and Perry, 1992). The cellular Rubisco concentration is directly related to the maximum rate of photosynthesis in higher plants and algae (Björkman, 1981; Rivkin, 1990; Orellana and Perry, 1992). From activity measurements of Rubisco, a linear relationship can be established to both actual and maximum photosynthesis as measured by $^{14}CO_2$-uptake (Fig. 2) although maximum Rubisco activity did not match maximum photosynthesis, and actual Rubisco activity was not related to photosynthesis at all (Mouget *et al.*, 1993). The Rubisco antibodies raised from *Chaetoceros gracilis* protein (Orellana and Perry, 1992) cross-reacted with 26 species of phytoplankton and immunofluorescence could be detected by flow cytometry (Orellana and Perry, 1995). However, in a number of tested species, immunostaining was inconsistent and only 26-35% of cells from field samples were labelled in this study. Incomplete permeabilization of cell membranes – crucial to allow the relatively large antibody molecules to pass through several membranes into the chloroplast – was claimed to be responsible for the limited success of the applied probe. Ethanol proved to be the only suitable fixative for this labelling approach, which will limit its application to field studies because chlorophyll autofluorescence necessary to differentiate phytoplankton from other particles is bleached by the alcohol treatment. Glutaraldehyde fixation prevented cell labelling.

The uptake of nitrate by phytoplankton is a central issue in biological oceanography due to its importance to primary production and vertical flux of biogenic carbon. For an oceanic ecosystem, the rate of "new" production (relying on external inputs of nitrate in contrast to "regenerated" production thriving on ammonium; Dugdale and Goering, 1979) directly relates to the sinking flux of biogenic material (Eppley and Peterson, 1979). Assimilatory nitrate reductases catalyze the first step of nitrate assimilation, the reduction of NO_3 to NO_2. Despite growing knowledge of nitrate reductase (NR) activity in phytoplankton (Berges, 1997), important aspects of NR regulation and control at the cellular and molecular level have not been addressed adequately (Gao *et al.*, 1993). Polyclonal antibodies against polypeptide sub-units provided cytometric detection and quantification of nitrate reductase in the marine diatom *Skeletonema costatum* (Jochem *et al.*, 2000). The extremely low abundance of this protein of less than 10 fg $cell^{-1}$ (Gao, 1997) demanded a secondary antibody approach for higher fluorescence yields. FITC-labelled anti-rabbit antibodies from goat were employed to mark the rabbit-raised primary anti-NR. Chlorophyll extraction further enhanced sensitivity. The immunolabelling provided high resolution of NR synthesis induction in ammonium-grown *S. costatum* within the first 24 hrs after inoculation into nitrate medium (Fig. 3a) and depicted the subsequent dynamics of this protein's abundance as the chemostat culture adapted to the change in nitrogen supply (Jochem *et al.*, 2000). Cytometric anti-NR fluorescence was linearly related to cellular NR abundance derived from densiometry of Western Blots (Gao, 1997) but showed much higher resolution at low concentrations (Jochem *et al.*, 2000; Fig. 3b). NR abundance as estimated by both flow cytometry and Western analyses of light-period samples exhibited a hyperbolic relationship to NR activity (Jochem *et al.*, 2000), suggesting post-translational activation of the NR protein (Smith *et al.*, 1992). Dark-period samples lacked any relationship between NR abundance and activity because NR activity is suppressed in the dark (Smith *et al.*, 1992; Gao, 1997).

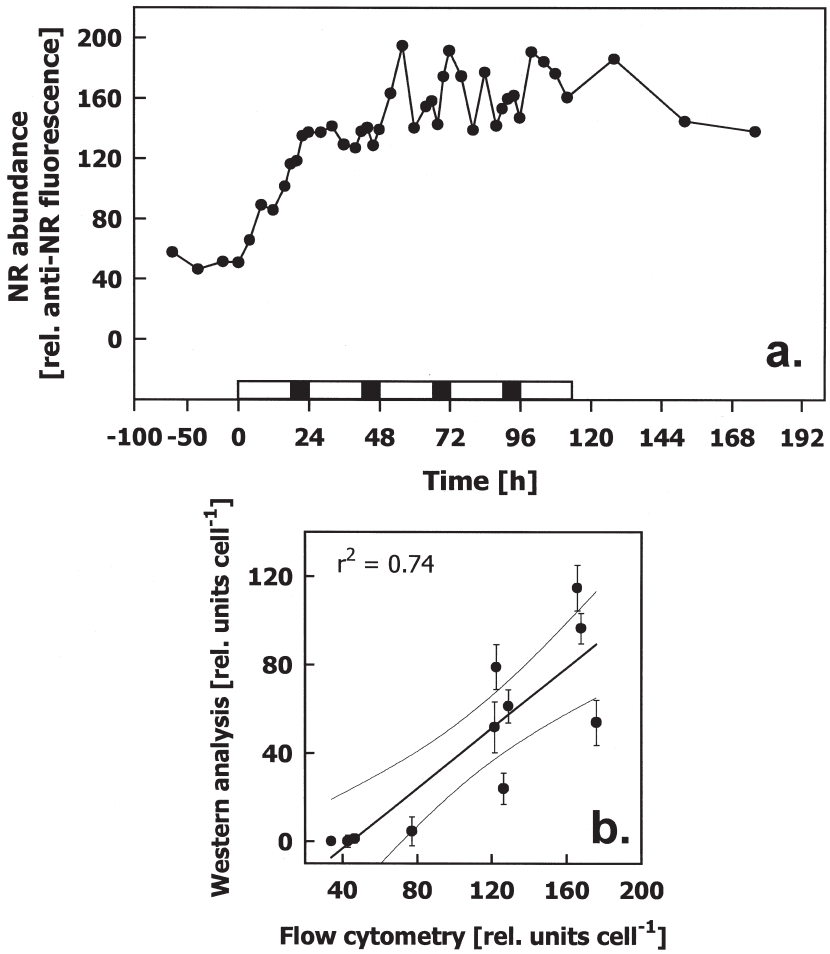

FIG. 3. – (a) Induction of nitrate reductase (NR; relative fluorescence units of anti-NR labelling) after a shift from ammonium to nitrate as nitrogen source (t=0) in the marine diatom *Skeletonema costatum*; light/dark cycle is indicated during the first 114 hrs after the nutrient shift when more than one daily sampling was performed. (b) Relationship between NR abundance as determined by densiometry of Western Blots and by immunolabelling and flow cytometry (both relative units) for mid-light period samples (r^2=0.74, p<0.001). From Jochem *et al.*, 2000.

The stickiness of diatom cells was assessed cytometrically by FITC-tagged concanavalin A, a lectin that binds to glucose and mannose on the cell surface (Waite *et al.*, 1995). The stickiness, defined as the probability that two colliding diatoms will remain attached, is one of the most important factors regulating aggregate formation (Dam and Drapeau, 1995). By altering the biomass size spectrum, aggregates in turn can influence the vertical flux of biogenic carbon (Smetacek, 1985; Villareal *et al.*, 1993) and change the availability of food to grazers (Stoecker *et al.*, 1981). Stickiness is believed to arise from the exudation of mucus, either present on the cell surface (Smetacek, 1985) or as transparent exopolymer particles (TEP) in seawater (Passow *et al.*, 1994; Dam and Drapeau, 1995). Concanavalin A fluorescence varied 5-fold among different diatom species and 2-3-fold within a given species in different physiological states (Waite *et al.*, 1995); for *Thalassiosira pseudonana* and *Chaetoceros neogracile*, cell surface sugar compounds increased with nitrate and silicate limitation in batch cultures. Although the concanavalin-A-FITC approach was applicable to formalin-fixed cells as well, half saturation time of the staining was longer (4.1 hrs vs. 0.2 hrs) and final fluorescence yield higher (0.05 vs. 0.03) in preserved as compared to live cells.

MOLECULAR PROBES FOR METABOLIC ACTIVITY AND NUTRIENT STATUS: KINETIC MEASUREMENTS

The principle of kinetic measurements is the application of non-fluorescent substrates that are processed by metabolic processes of interest with-

in the cells, cleaving the applied molecule and releasing a strong fluorigen that has to exhibit low leakage from the cells. Hydrophilic dyes such as fluorescein or FITC have proved useful but others are in use as well.

The cleavage of the non-fluorescent substrate results in an accumulation of fluorescence within the cell that initially increases until saturation. In contrast to fluorescence microscopy, flow cytometry cannot only distinguish labelled from non-labelled cells, but can estimate the fraction of the population with a positive reaction on the applied substrate. It can also provide a quantitative measure, in terms of relative fluorescence, of the rate at which the cells process the substrate. Quantitative measurements must, therefore, be taken either after saturation of fluorescence accumulation or at a specific time-point after substrate addition that is standardized for all samples to be compared.

Fluorescein diacetate, originally introduced to differentiate metabolically active from inactive bacteria in soils (Schnürer and Roswall, 1982), water (Holzapfel-Pschorn *et al.*, 1987) and marine sediments (Köster *et al.*, 1991; Gumprecht *et al.*, 1995), was shown to be applicable to the study of phytoplankton physiology as well. FDA is a nonpolar, nonfluorescent substance, which enters the cells freely. Inside the cell, nonspecific esterases, among them lipase and acylase but not acetylcholinesterase (Guilbaut and Kramer, 1966), break the FDA molecule into one brightly fluorescing fluorescein and two acetate molecules. Being highly polar, the fluorescein is trapped within cells exhibiting cell membrane integrity and the amount of fluorescence therefore increases over time depending on the metabolic activity of the esterases. The FDA enzyme assay has recently been complemented with a protocol for cryopreservation of labelled phytoplankton (Faber *et al.*, 1997) that will greatly improve the processing of large sample sets and the application in field studies.

The esterases involved in the FDA assay turn over on a time frame of several hours (Yentsch *et al.*, 1988). Therefore, this technique seems appropriate to detect changes in metabolic activity on a day-to-day or even shorter basis, which makes it well suited to monitor short-term phytoplankton responses to environmental changes and pollution. Reduced esterase activity in terms of FDA fluorescence accumulation in *Selenastrum capricornutum* was documented after exposure to high (450 ppb) concentrations of copper, whereas lower concentrations induced an increase in esterase activity; 27 hrs into the incubation, esterases resumed their initial performance irrespective of Cu concentrations (Arsenault *et al.*, 1993). Using flow cytometry, Geary *et al.* (1998) distinguished between cyanobacteria, *Microcystis aeruginosa*, grown under different light intensities or under phosphate replete/deplete conditions for 2 days; the light-covarying FDA fluorescence corresponded to different growth rates. Covariation of FDA fluorescence and maximum photosynthetic carbon fixation with cell volume was reported from 8 phytoplankton species ranging about 2 to 15 μm in size (Dorsey *et al.*, 1989). The regulation of metabolic activity in phytoplankton upon prolonged darkness was also assessed by FDA (Dorsey *et al.*, 1989; Jochem, 1999). The latter study revealed two distinct survival strategies among phytoflagellates (Fig. 4): One group of species adjusted to darkness by reducing their metabolic activity within a few days; they were able to sustain their population abundance and resume rapid

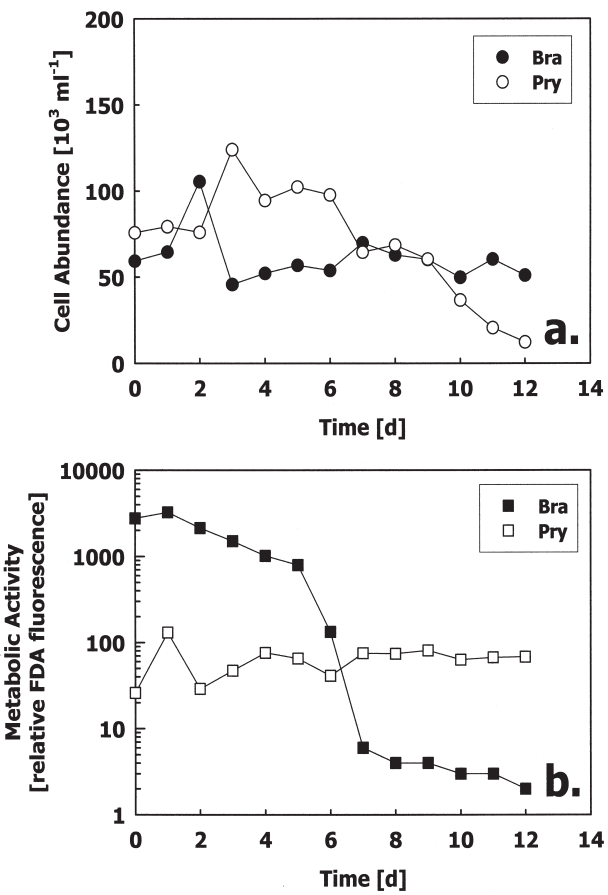

FIG. 4. – Dark survival strategies in two phytoflagellates. (a) Cell abundance (10^3 ml^{-1}), (b) cellular metabolic activity in terms of mean FDA fluorescence (rel. units). Bra = *Brachiomonas submarina*, Pry = *Prymnesium parvum*. After Jochem, 1999.

growth after re-illumination. The other group exhibited no change in metabolic activity upon darkness and seemed to run out of energy; these species could not sustain their cell numbers in the dark and did not resume growth after re-illumination.

Changes in peroxidase activity in the marine diatom *Phaeodactylum tricornutum* exposed to copper pollution were monitored by dihydroethidium, also called hydroethidine (Cid *et al*., 1996a). The oxidation of the initially blue fluorescent dye by peroxidase in the presence of reactive oxygen species (peroxide or superoxide) within the cell results in a brightly red fluorescent ethidium. In the presence of chlorophyll autofluorescence, the authors monitored the red peroxidase activity fluorescence in the FL2 channel (560-590 nm) of a Becton-Dickinson FACScan after 30 min incubation with the dye. Cytometric analysis revealed an increase in peroxidase activity after 48 hrs and a subsequent decrease to initial levels after 96 hrs of copper exposure.

A new family of fluorogen-bound enzyme substrates called ELF-97™ (Enzyme Labelled Fluorescence; Molecular Probes, Eugene, OR) provides a powerful tool for individual phytoplankton analysis in complex field assemblages (Larison *et al*., 1995; Haugland, 1996). The ELF substrate is soluble and low fluorescent. Hydrolysis of the substrate results in the precipitation of an insoluble fluorescent product with excitation maximum at 365 nm and green fluorescence emission (515 nm; Haugland, 1996).

ELF alkaline phosphatase substrate (Molecular Probes, Eugene, OR) proved useful in the study of the physiological status of phosphorous nutrition in phytoplankton (González-Gil *et al*., 1998; Dyhrman and Palenik, 1999). Phosphate-limited cells could be clearly distinguished from unlimited cells in culture and field samples, and alkaline phosphatase activity could be cytometrically quantified in dependence of phosphate concentration in algal cultures (González-Gil *et al*., 1998). Cells were incubated in a commercially available ELF buffer for 30 mins at room temperature in the dark and then washed in 10 mM phosphate saline buffer (PBS); thus treated preparations kept their ELF fluorescence over 3 months dark storage at 4°C (González-Gil *et al*., 1998). ELF represents an "off-the-shelf" probe of P starvation that provides the same information that can be obtained by molecular probes or antibodies for cell-surface proteins involved in P-uptake (Dyhrman and Palenik, 1995). In contrast to antibodies with mostly specific cross-reactivity with only one or a group of some closely related species, ELF is applicable to a wide variety of species (González-Gil *et al*., 1998) though potentially not for all; namely diatoms were scarcely labelled in field samples in which dinoflagellates showed bright labeling (Dyhrmann and Palenik, 1999).

Higher ELF fluorescence per cell in *Isochrysis galbana* as compared to the much larger *Amphidinium* sp. could not be related to alkaline phosphatase activity as revealed by other methods (González-Gil *et al*., 1998). The authors conclude that higher fluorescence in *I. galbana* was due to higher affinity of its phosphatase for ELF. Thorough intercalibration is still required for quantitative comparison of phosphatase activity in different species using ELF.

The manufacturer states that ELF fluorescence is detectable by 488 nm blue light excitation used in benchtop cytometers (Haugland, 1996), which was confirmed by Pecorino *et al*. (1996). However, Dyhrman and Palenik (1999) were not able to track ELF fluorescence using 488 nm excitation but were successful with 363 nm excitation. Cytometric detection of ELF in phytoplankton was performed by UV excitation as well (FACS Vantage, 100 mW Innova laser, 333-364 nm; González-Gil *et al*., 1998). The positive results under blue light excitation (Pecorino *et al*., 1996) might be related to the lack of autofluorescence in the studied cells. So far it seems that ELF-97™ substrates, which include probes for lipase, sulfatase, esterase and others (Haugland, 1996), might remain restricted to more expensive and powerful machines carrying a UV laser.

OUTLOOK

Development of molecular probes for a variety of ecologically significant physiological processes have progressed well over the last few years. Today, molecular probes to detect nitrogenase activity, iron limitation, copper pollution, and phosphate stress or to discriminate between active and inactive cells are already tested in field samples.

To raise antibodies in mammalian hosts, the antigen of interest should be of a proteinaceous nature, either a structural protein of the cell or an enzyme. In cases where the target of interest is a non-protein, it can be bound to carrier proteins. For example, by binding to bovine serum albumin (BSA), antibodies against brevetoxin, the toxic multi-ring polyether produced by the harmful

dinoflagellate *Ptychodiscus brevis* (Baden *et al.*, 1984, 1988), and tetrahydropurine-based dinoflagellate toxins causing paralytic shellfish poisoning (Guire *et al.*, 1988) were raised. Antibodies against the amnesic shellfish poisoning toxin domoic acid were produced by ovalbumin binding (Smith and Kitts, 1995). None of those antibodies has been applied to flow cytometry yet but potentially provide tools to assess the cellular toxin concentration in dinoflagellates and its variation with environmental factors. This is especially promising where toxic species can be marked by taxon-specific antibodies (Peperzak *et al.*, 2000).

Specific cell surface proteins expressed upon phosphate depletion have been identified in the chlorophyte *Dunaliella tertiolecta* (Graziano *et al.*, 1995) and *Synechococcus* sp. (Scanlan *et al.*, 1997). Antibodies raised against the latter also cross-reacted with *Prochlorococcus* sp. and potentially such antibodies might prove useful in detecting phosphate stress by flow cytometry in mixed populations. The expression of flavodoxin instead of ferredoxin, detected by specific polyclonal antibodies, was shown to reveal iron limitation in marine phytoplankton (LaRoche *et al.*, 1995, 1996). And ntcA, a transcriptional activator that regulates the transcription of a series of genes encoding proteins for nitrate uptake and assimilation in *Synechococcus* sp., has recently been isolated and sequenced (Lindell *et al.*, 1998); ntcA was suppressed by ammonium, whereas it was supported by nitrate as nitrogen source and it might eventually provide a tool to detect nitrate versus ammonium as principle nitrogen source of phytoplankton growth.

Further biomarkers for ecologically and environmentally significant processes in marine organisms are to be developed and tested in laboratory and field studies. Fluorescent dyes for cell constituents (static measurements I), commonly used in histochemistry, are hitherto largely underused in aquatic research and flow cytometry. Only the different dyes for DNA labelling gained much interest by researchers using aquatic cytometry. Otherwise, cytometric application of such dyes is still restricted to limited and isolated laboratory studies. It can be assumed, however, that this group of dyes can provide a much wider, yet unexploited palette of applications, which can demonstrate changes in cellular components upon environmental changes and differences in responses among different species. Such results will enhance our understanding on how biodiversity is sustained and phytoplankton flourishes in oceans and lakes on a cellular level. The ease of use of such dyes as compared to more complex preparations involved in immunolabelling (Peperzak *et al.*, 2000) makes further development and investigation of fluorescent static measurements desirable.

Much more effort was directed towards the development of molecular probes during the last decade. However, a lot of these applications still rely on gels and blots. Although each development of molecular tools starts from gels and blots, these techniques are hardly quantitative and too laborious for routine sample monitoring. Transferring these molecular tools to rapid and easy to use techniques for routine application in environmental monitoring and ecosystem studies will provide a valuable step for research in the new century. Such techniques may include immobilized antibodies or other biomolecules on the surface of optical filters, fibers or other surfaces suitable for later optical detection, and flow cytometry.

A major problem of present molecular probes is their specificity. Although highly specific antibodies are sometimes desired, there is a vast field of applications and scientific questions that would prefer a more universal probe. Nitrate reductase antibodies, for example, may prove useful to investigate phytoplankton nitrogen dynamics, a key question in biological oceanography. The hitherto applied antibodies cross-react, however, only with dinoflagellates (Fritz *et al.* 1996) or centric diatoms (Gao, 1997; Jochem *et al.*, 2000). Antigenecity of NR from different phytoplankton species seems divergent (Gao *et al.*, 1993), and despite homologous amino acid sequences corresponding to redox centers the overall similarity in amino acid sequences from different plant and fungal NR is less than 50% (Campbell and Kinghorn 1990). The generation of immunoprobes against widely conserved peptide sequence domains offers one approach for generic immunoassays. However, the observation that antibodies targeting single epitopes may not yield sufficient signal for detection of low abundant targets, such as NR (Jochem *et al.*, 2000), requires further development of high fluorescence yield fluorochromes such as the Alexa® series (Haugland, 1996).

Whereas, for example, the ELF™ substrates for alkaline phosphatase are already commercially available and ready for use in routine applications, most of the other molecular probes are still in the development and testing phase in separate research laboratories. It can be assumed that the development of new probes and techniques will remain a primary

responsibility of research laboratories. A fast and efficient technology transfer for commercial production and distribution is necessary, however, to provide new probes and technologies in sufficient quantity to allow for a wide and routine application in aquatic ecology, so that their promises can be exploited efficiently and broadly. Since numerous probes may be directed towards environmental monitoring and water quality control and may open a wide market, given the ease and time-saving of cytometric analyses, commercial participation in probe development should be encouraged for rapid production and distribution of such new probes.

Since the environment directly affects cells, not populations, the measurement of individual cells is desirable. In addition to differences among separate species in physiological capacities such as, for example, photosynthetic efficiency, microenvironments such as nutrient patches and biological factors such as cell cycle stage might cause non-uniform distributions of properties among the cells of a single species population (Olson and Zettler, 1995). Cytometric analysis of variable chlorophyll fluorescence (Olson and Zettler, 1995; Olson *et al*. 1996, 1999), therefore, most valuably complements bulk measurements by fluorometer-based pump-and-probe systems. This technique provides a powerful tool to assess the regulation of phytoplankton growth and to estimate the contributions of different phytoplankton groups to total primary productivity (Olson *et al*., 1999). But today only one self-modified instrument is set up to provide such measurements. For wide application in ecological research, the necessary technical modifications to commercial flow cytometers should be commercially available as well. Since such applications are off the main stream of major commercial manufacturers, which primarily direct their developments to medical research and diagnostic, small business solutions might provide better support for the special needs of oceanography. Again, fast and efficient technology transfer from academically based research and development to such small businesses should be encouraged.

Over all, recent developments in cytometry instrumentation and phytoplankton probes have provided tools for much deeper insights into processes that govern plankton development in response to environmental conditions on a single-cell basis. More progress is steadily achieved, and probing the physiological state at the single-cell level by flow cytometry seems still in its infancy.

ACKNOWLEDGEMENTS

The Nancy Lee and Perry Bass Endowment to the University of Texas at Austin Marine Science Institute supported preparation of this manuscript.

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