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Estimation of primary production by flow cytometry

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Flow cytometry (FCM) is a method for the rapid quantitative analysis of fluorescence and size-related characteristics of single cells. It is well suited for examining the phytoplankton component in marine microbial assemblages because of the distinctive red fluorescence emitted by chlorophyll. The use of FCM in phytoplankton ecology is relatively recent and studies to date have focused on the spatial and temporal distributions of various cell groups. The unique advantage of FCM lies in its ability to analyse large numbers of individual cells. Given this, FCM offers the potential to recover the bulk production of phytoplankton from an analysis of the constituent flora. To date, various indices of growth have been estimated for oceanic cyanobacteria, prochlorophytes, and other groups easily detected by FCM. However, the broader goal of estimating overall primary production by FCM has been achieved only in certain circumstances. To this end, efforts are being made to use FCM to study phytoplankton population growth, cell division, viability, and photosynthetic activity. These studies are discussed here under the headings of cell counting methods, cell staining methods, chlorophyll fluorescence methods, and cell sorting methods.

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Introduction

Flow cytometry (FCM) is a method for the rapid, quantitative analysis of fluorescence and size-related characteristics of single cells (Melamed *et al.*, 1990). It is well suited for examining the phytoplankton component in marine microbial assemblages because of the distinctive red fluorescence emitted by chlorophyll, which is present in all phytoplankton. In less than a decade, the field of oceanographic flow cytometry has developed from one of modest exploratory studies (Olson *et al.*, 1983; Yentsch *et al.*, 1983) to one encompassing a wide range of applications that have yielded important and hitherto unattainable information on marine phytoplankton (Chisholm *et al.*, 1988a; Yentsch and Horan, 1989; Demers, 1991).

The fundamental feature that distinguishes FCM from many other techniques used in phytoplankton ecology is that measurements are made on individual cells rather than on the bulk photoautotrophic biomass. This feature makes it possible, in principle, to attempt a recovery of bulk properties from knowledge of the constituent properties (Platt, 1989). Furthermore, this feature

allows examination of cell-to-cell variation, which is the basis for integrating studies at the organism level with studies of the ecology of phytoplankton assemblages (Platt, 1989). Whilst it is true that fluorescence microscopy, image cytometry, and Coulter counting also provide information on individual cells, FCM offers important and unique features unavailable in these other techniques.

A recent general review has summarized the advances achieved in the first decade of aquatic flow cytometry with indications of future directions (Olson *et al.*, 1991). The present review will have a more restricted scope. The aim of this paper is to survey various ways in which FCM might be used to study the biological processes underlying oceanic primary production. It is now possible to estimate various indices of growth for oceanic cyanobacteria, prochlorophytes, and other groups easily detected by FCM (i.e., numerically abundant groups). However, the broader goal of estimating overall primary production by FCM has been achieved only in certain circumstances. To this end, efforts are being made to use FCM to study phytoplankton population growth, cell division, viability, and photosynthetic activity (Yentsch

and Campbell, 1991). These studies are discussed here under the headings of cell counting methods, cell staining methods, chlorophyll fluorescence methods, and cell sorting methods.

Cell counting methods

The flow cytometer is an excellent tool for counting numerically abundant autofluorescent particles at sea (Olson *et al.*, 1985, 1988, 1990a, b; Chisholm *et al.*, 1988b; Li and Wood, 1988; Børshheim *et al.*, 1989; Li, 1989; Vaultot *et al.*, 1990; Veldhuis and Kraay, 1990; Yunev *et al.*, 1990; Li *et al.*, 1992a, b, 1993). In fact, it is the only method available that can give near real-time estimates for the abundance of oceanic prochlorophytes whose weak fluorescence makes detection by microscopy an arduous task. With most conventional flow cytometers, it is difficult to count cells that occur at concentrations less than 10^2 – 10^3 ml⁻¹. This limitation is imposed by the volume of sample that can be analysed within a reasonable length of time. Higher rates of sample throughput have been achieved (3.3–10 ml min⁻¹), but only in user-customized instruments (Dubelaar *et al.*, 1989; Olson, 1989). A single instrument that incorporates the advantages of high sensitivity (required for picoplankton) and of high sample throughput (required for nano- and microplankton) would be a great asset in biological oceanography.

In using FCM as an efficient means for counting particular phytoplankton groups (e.g. picoplanktonic cyanobacteria and prochlorophytes, cryptomonads and other autofluorescent nanoplankters), it is possible to estimate rates of population growth simply by monitoring changes over time. Observations may be (i) made on fresh samples collected from the ocean at various sampling frequencies (e.g. hourly, daily) or (ii) made after some time (or several times) on a sample enclosed in a bottle and incubated *in situ*, on ship deck, or in environment-controlled chambers.

In the first protocol of observing changes in nature, we see only the net result arising from the underlying processes of reproduction, grazing, mortality, sinking, motility, turbulent diffusion, and advective transfer. This protocol, being free of experimental manipulations, is elegantly simple. The results of Waterbury *et al.* (1986), obtained not by FCM but by epifluorescence microscopy, illustrate the utility of this approach for the cyanobacterium *Synechococcus* for sampling time scales of hours, days, and weeks. An example illustrating the use of FCM for monitoring population size in a water parcel (Fig. 1) shows differential rates of change for *Synechococcus*, prochlorophytes, and photosynthetic eukaryotes (<10 μ m). On a negative note, the implementation of this protocol may sometimes prove

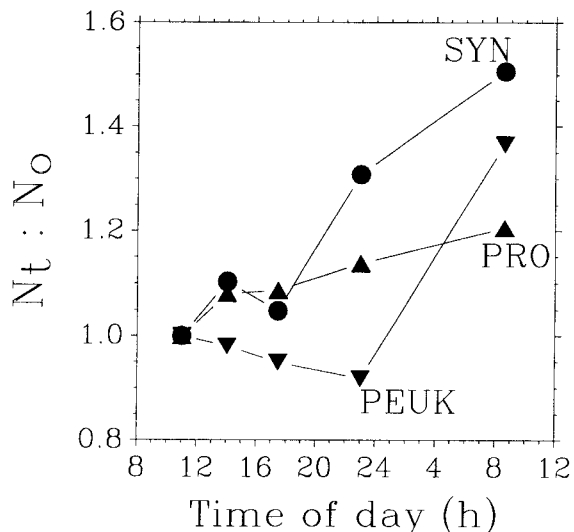


Figure 1. Differential growth rates of *Synechococcus* sp. (SYN), prochlorophytes (PRO), and photosynthetic eukaryotes (PEUK) indicated by the ratio of counts at time t (N_t) to counts at time zero (N_0). Observations were made by repeated sampling of a water parcel indicated by a drifting sediment trap array (40°4.20'N 47°12.40'W to 39°56.60'N 47°4.70'W, 2–3 May 1989).

difficult. Aside from the practical considerations of dedicating ship-time to a Lagrangian study, there is often uncertainty that patchiness may exist at the scale of ship-to-drifter distance.

Under certain circumstances, Lande *et al.* (1989) suggested that it may be possible to estimate *in situ* net population growth rate (reproduction minus overall mortality) without the need for repeated observations. This proposition is based on the idea that time-change in population abundance is due to the effects of net growth (reproduction minus mortality), sinking plus motility, turbulent diffusion, and advection. If conditions exist where it can be assumed (or demonstrated) that there is no change in population size with time, that sinking and motility rates are negligibly small, and that advection is unimportant, then the net growth rate can be inferred from depth profile measurements of phytoplankton abundance and turbulent diffusion (Lande *et al.*, 1989).

The second protocol, where cell counts are made on incubated samples, obviates the need to consider sinking, motility, diffusion, and advection. However, it is conceivable that artifacts may occur when plankton are enclosed in small volume containers (Carpenter and Lively, 1980). The attractiveness of this experimental approach is that incubations can be executed in various ways, and the plankton can be manipulated as desired; this protocol offers a way to perform direct tests of many important hypotheses. A simple implementation is to

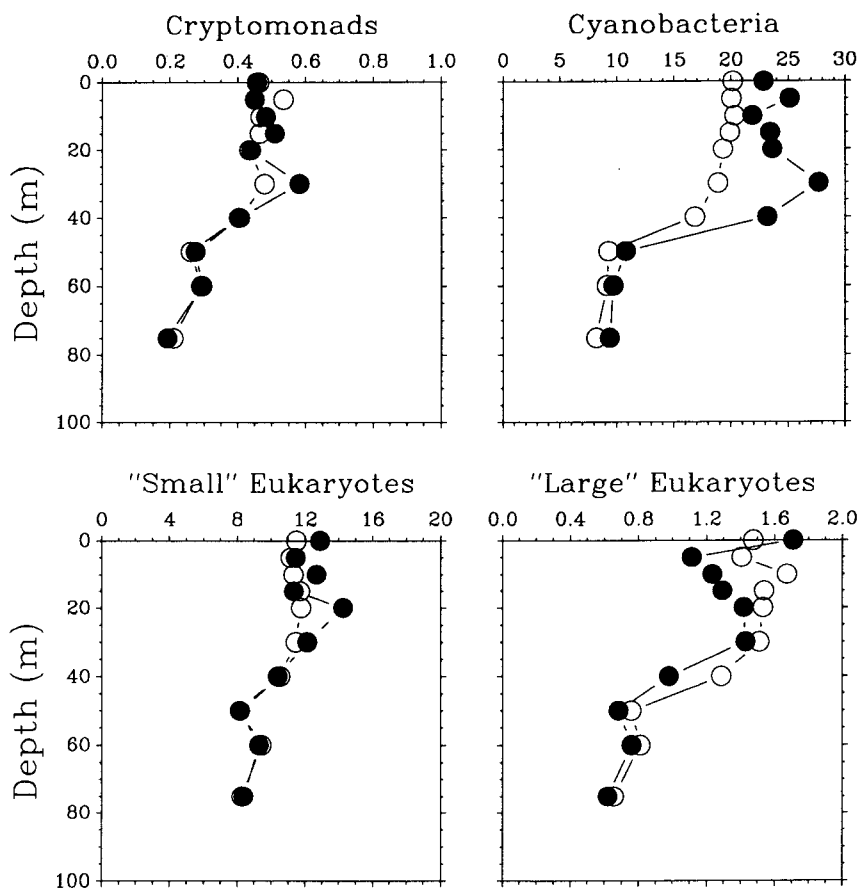


Figure 2. Differential growth rates of cryptomonads, cyanobacteria, "small" eukaryotes ($<2 \mu\text{m}$), and "large" eukaryotes ($>2 \mu\text{m}$). Cell counts were made at the beginning (○) and end (●) of a 12 h incubation (07.30–19.30 h) under *in situ* conditions ($40^{\circ}14.95'N$ $64^{\circ}35.16'W$, 15 April 1991). Units for population size are $10^3 \text{ cells ml}^{-1}$.

measure the change that occurs in samples incubated under *in situ* (Fig. 2) or simulated *in situ* conditions.

Useful variations include incubations at different light and temperature regimes to examine photo- and thermal adaptive responses; nutrient additions to test for nutrient limitation; selective metabolic inhibition, differential size fractionation, or dilution with filtered sea water to inactivate, remove, or diminish grazing pressure (Li, 1986). Under these experimental conditions, FCM measurements of cell abundance, cell size, and pigment fluorescence of individual cells often indicate changes that cannot be inferred from measurements of extracted bulk chlorophyll concentration or total filterable ^{14}C activity.

Cell staining methods

Cell volume, light scatter characteristics, and autofluorescent pigment content are inherent properties of the

cell used in the counting methods described in the previous section. Additionally, other properties of cells may be studied using staining reagents. These latter properties can be viewed as being structural or functional. Structural properties are those concerning the contents and physical states of chemically defined entities (e.g. DNA, lipids); functional properties refer to activities that undergo physiological changes (e.g. enzyme activity). In the context of primary production measurements, a pragmatic goal is to relate FCM-measured stained fluorescence to the rate of primary production or population growth. The use of fluorescent probes in FCM to study phytoplankton cells is still at an early stage; but recent work on the cellular contents of DNA, membrane lipids, and ribulose biphosphate carboxylase, as well as on esterase activity appear promising.

One avenue that leads to estimates of algal production is through investigation of the cell division cycle. The relationship between population growth rate and the

fraction of cells undergoing mitosis was clearly delineated by McDuff and Chisholm (1982) and extended by Vaultot (1992). Recently, Chang and Carpenter (1988a) broadened the concept to enable population growth to be estimated from measurements of DNA content relating to any terminal event (*sensu* Mitchison, 1971) in the cell cycle. Using microfluorometry to establish frequency distributions for cells whose DNA were stained with DAPI, Chang and co-workers have been able to verify their method (Fig. 3A). The recent success in using FCM to establish DAPI-DNA frequency distri-

butions for oceanic phytoplankton (Boucher *et al.*, 1991; Vaultot and Partensky, 1992) points to probable rapid progress in an FCM implementation of the Chang-Carpenter method for phytoplankton in nature.

A novel FCM method that monitors cell division has recently been described by Yentsch and Campbell (1991). A vital fluorescent stain (PKH-2) is used to label the lipid component of cell membranes. After cell membranes are loaded with PKH-2, there is no loss of stain fluorescence until the cell divides. When division occurs, each daughter cell then contains approximately half the

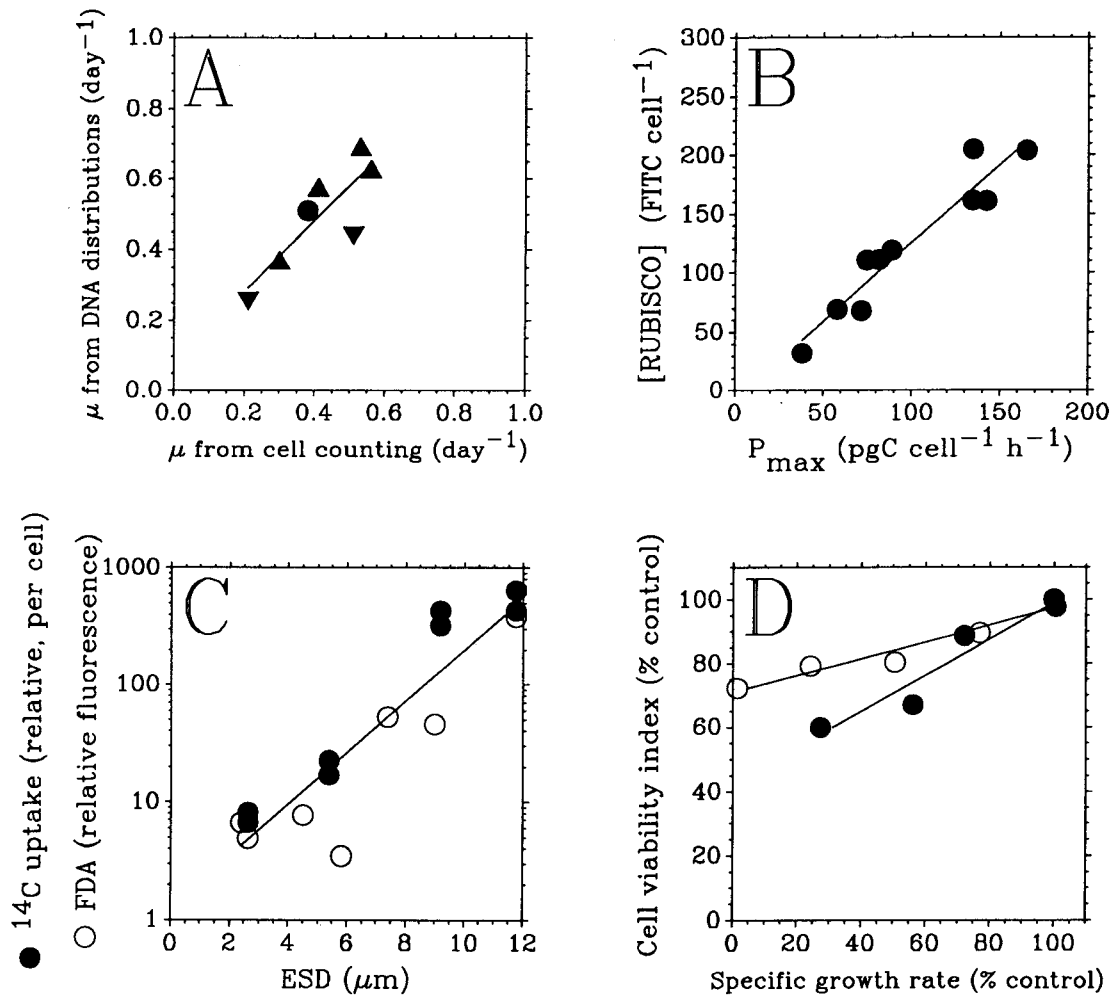


Figure 3. A. DNA method of Chang and Carpenter. Specific growth rate of various algae estimated by the DNA distribution method (microfluorometry) versus growth estimated by conventional counts of population size over incubation time. Different symbols identify data from Chang and Carpenter (1988b, ●), Antia *et al.* (1990, ▲), and Chang and Carpenter (1991, ▼). B. Rubisco method of Orellana and Perry (1992). Relative fluorescein fluorescence per cell versus light saturated rate of photosynthesis in *Thalassiosira weissflogii*. C. FDA method of Dorsey *et al.* (1989). Dependence of fluorescein fluorescence (○) and ¹⁴C uptake per cell (●) on cell size (equivalent spherical diameter, μ m) for eight algal species. D. FDA method used to assess toxicity of sodium dodecyl sulfate (●) and atrazine (○) on *Selenastrum capricornutum* (Gala and Giesy, 1990). Cell viability index is the mean fluorescein fluorescence of viable cells multiplied by the number of viable cells. Data shown are averages of responses after 4, 24, and 48 h exposure to the toxicants.

fluorescence per cell of the mother. This stain was developed for use in animal cells (Horan and Slezak, 1989) and the pattern of 50% reduction has been followed for up to eight cell divisions. The advantage of the PKH-2 method (compared to say DNA labelling methods) is that each generation can be identified by its signature of fluorescence intensity. At present, the use of PKH-2 in studies of phytoplankton division is hampered by staining variabilities: Cucci *et al.* (1992) think that perhaps this stain may only be suited for certain phytoplankton groups or only at particular cell cycle stages.

A powerful staining method for examining primary production of single cells has been developed from immunological principles by Orellana *et al.* (1988). The method rests on the foundation that ribulose biphosphate carboxylase (Rubisco) is the enzyme responsible for net CO₂ fixation in autotrophs, and that there is a direct relationship between the concentration of this enzyme and the maximum rate of photosynthesis. By developing an antiserum to Rubisco (e.g. in rabbits injected with *Chaetoceros* Rubisco), it is possible to use immunofluorescence methods (e.g. FITC conjugated goat anti-rabbit IgG) to report on Rubisco in test species of algae. Using FCM to quantitate FITC fluorescence in *Thalassiosira weissflogii*, Orellana and Perry (1992) demonstrated a strong correlation between the presumed level of Rubisco per cell and photosynthetic capacity per cell (Fig. 3B).

In a fourth staining method, rates of photosynthesis and growth are examined by correlation with "cell viability" (Dorsey *et al.*, 1989). Here, viability is defined as intracellular esterase activity capable of cleaving fluorescein diacetate (FDA) to give fluorescein. This method has the premise that esterases, which are essential to phospholipid turnover in cell membranes, are more active in healthy, rapidly growing cells than in stressed cells. The pivotal feature of this method is that FDA is non-fluorescent, but fluorescein (into which FDA is converted in the presence of esterase) is fluorescent. In a study of several laboratory algal cultures, Dorsey *et al.* (1989) demonstrated a good correlation between fluorescein fluorescence intensity per cell and photosynthetic ¹⁴C uptake per cell (Fig. 3C). The FDA method has also been applied successfully in an FCM assessment of toxicity response (Fig. 3D).

For applications to be robust, fluorescent probes should be used with a clear understanding of the underlying physiological processes. In this regard, a distinction might be made between, on the one hand, DNA and Rubisco, whose intracellular roles are well understood, and, on the other hand, PKH-2 and FDA, whose functions and controls at the intracellular level are less well understood.

Chlorophyll fluorescence methods

The relative ease with which chlorophyll fluorescence can be detected by FCM makes it possible to implement, on an individual cell basis, a number of the methods currently used to probe assemblage photosynthesis from measurements of bulk fluorescence (Falkowski and Kiefer, 1985). The FCM versions of two such methods, DCMU enhancement of fluorescence and the pump-and-probe flash technique, are able to yield information concerning individual cell responses which is not recoverable from the corresponding bulk methods. However, interpretation of results from the FCM methods must be undertaken with at least the same degree of caution as with conventional results because the same biophysical principles, of which we have incomplete knowledge, apply.

When phytoplankton are exposed to light, the photons arriving at Photosystem II can either be used to drive photochemical reactions, or the photons can be dissipated as fluorescence or heat. As a first approximation, we can regard fluorescence and PSII photochemical reactions as competing for excitation energy within the antennae Chl of PSII traps. The primary electron acceptor in PSII is the plastoquinone "Q". When Q is oxidized, the open PSII traps are able to absorb photon energy and thus fluorescence is quenched. However, when Q is reduced, photons arriving at the closed traps must be dissipated as fluorescence. To a first approximation, there is therefore an inverse relationship between fluorescence yield and photochemical reactions.

Recognizing this inverse relation, it is possible to maximize the variable fluorescence yield by minimizing the photochemical reactions. The herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) accomplishes this by blocking electron transport from the primary acceptor Q. Thus, the variable amount of fluorescence induced as a result of DCMU action is related to the capacity for photosynthesis. The proposal by Samuelsson and Öquist (1977) that DCMU-enhanced fluorescence may be used to study photosynthetic capacity has led to numerous successful applications in ecological studies of phytoplankton.

Recently, Furuya and Li (1992) described the use of FCM to measure DCMU-enhanced fluorescence in single cells. A crucial factor that determined the degree of enhancement was the intensity of the exciting beam. Cullen *et al.* (1988) showed that in commonly used flow cytometers the maximum excitation irradiances are many orders of magnitude higher than in conventional fluorimeters used for bulk measurements (e.g. Turner Designs). When the excitation intensity is low, photochemical reactions can keep up with the rate at which

photons arrive at the PSII centres: the fluorescence yield is thus at a minimum (F_0). However, when the excitation intensity is high, more energy is dissipated as fluorescence and the yield increases to a maximum (F_{max}). In this situation, the degree to which fluorescence can be further enhanced by the addition of DCMU becomes correspondingly less. Neale *et al.* (1989) showed that in an EPICS V flow cytometer (Coulter Electronics) operated at 250 mW excitation at 488 nm, the effective fluorescence was intermediate between F_0 and F_{max} . They concluded that the information derivable from FCM fluorescence depends strongly on instrument configuration. In using FCM to examine DCMU enhancement, it is therefore important to reduce the excitation intensity as much as possible. However, this imposes a limitation, yet to be solved, in the kind of cell that can be studied by this method.

The limitation arises because small phytoplankton cells, with their low chlorophyll content, fluoresce very weakly on an individual basis. The reduction of excitation intensity, which is necessary to achieve DCMU enhancement, effectively places the weakly fluorescing cells below the level of instrument detection. Nevertheless, for cells that can be detected at low excitation intensities, the expected correlation between fluorescence enhancement per cell and photosynthetic capacity per cell has been verified (Furuya and Li, 1992).

A useful index for the quantification of DCMU-enhanced fluorescence is the fluorescence response index (Cullen and Renger, 1979):

$$FRI = \frac{F_{DCMU} - F_{control}}{F_{DCMU}}$$

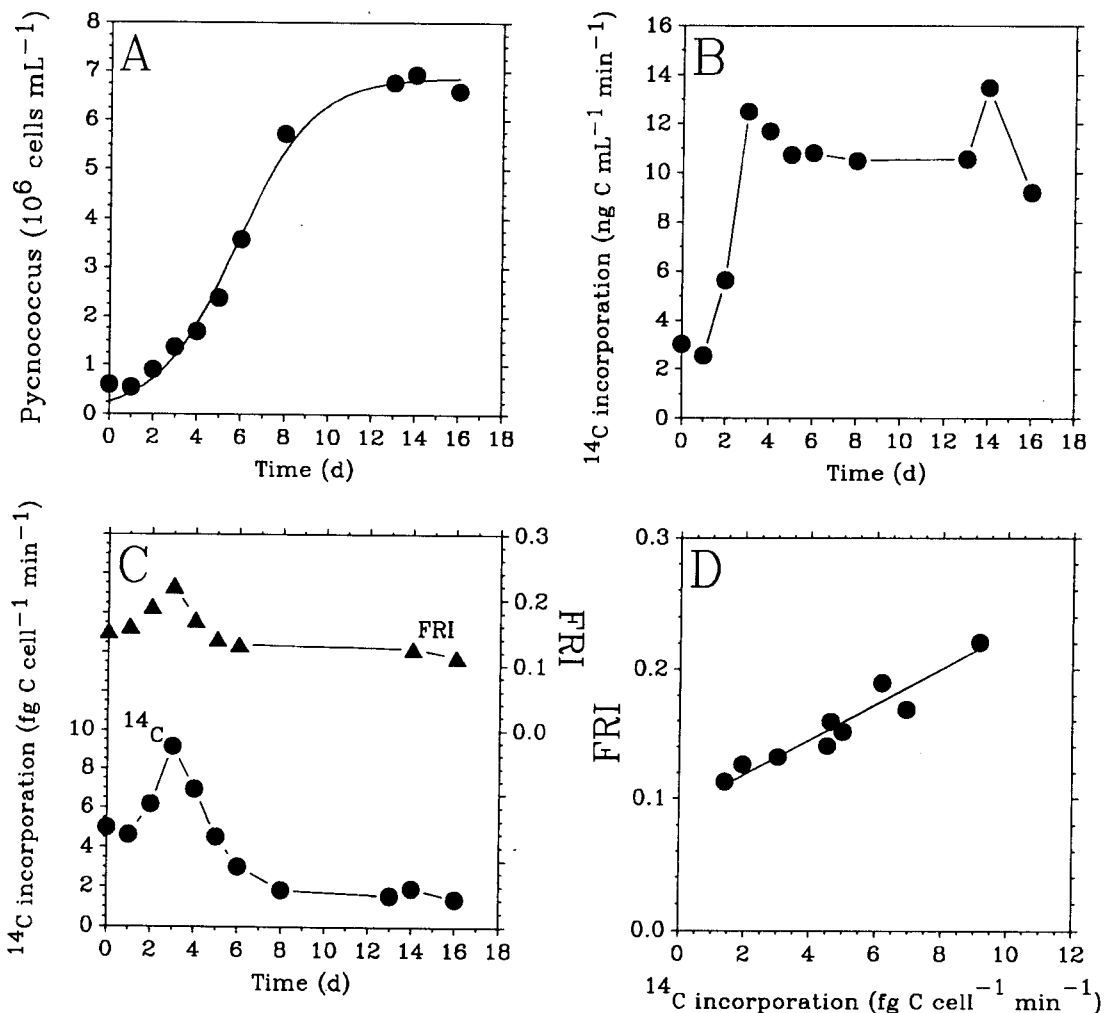


Figure 4. *Pycnococcus provasolii* in unialgal batch culture. A. Cell concentration. B. Rate of ¹⁴C incorporation per unit volume of culture. C. Rate of ¹⁴C incorporation per cell (●) and FRI (▲). Cells were dark-adapted for 5 min prior to analysis; DCMU was used at a concentration of 10 μM. D. Correlation of FRI and rate of ¹⁴C incorporation per cell.

This quantity may also be termed the cellular photochemical capacity (Vincent, 1981). In FCM application, F_{DCMU} is the mean fluorescence per cell measured after addition of DCMU to the sample; and $F_{control}$ is the mean fluorescence per cell measured in the no-addition control. In the common oceanic ultraphytoplankter *Pycnococcus provasolii* (Guillard *et al.*, 1991), there is a strong correlation between light-saturated rates of ^{14}C incorporation per cell and the FRI measured in a FACS Analyzer (Becton Dickinson) using minimal excitation (485 nm) from a mercury-cadmium arc lamp (Fig. 4).

The utility of the FCM method lies in its ability to measure FRI of individual species in algal mixtures or natural assemblages. An illustrative experiment (Fig. 5) shows the differential time-change of FRI for three different species cultured together in one flask (tri-culture) in comparison to the same species cultured separately in three other flasks (uni-cultures). For

Phaeodactylum tricornutum, whose growth pattern was similar in uni- and tri-cultures, the values of FRI were also very similar, indicating that the cellular expression and experimental measurement of FRI were not compromised by the presence of other species in the tri-culture. In contrast, the development of *P. provasolii* was very different in uni- and tri-cultures after the initial few days: this difference was reflected in high FRI values for the growing uni-culture, but low FRI values for the non-growing tri-culture.

In a study of the spring bloom in a coastal marine embayment, Furuya and Li (1992) were able to document temporal variations of the FCM-measured FRI of dominant diatoms and cryptophytes. Other experiments (not shown) indicate that the relationship between FRI and photosynthesis varies among species and growth conditions (e.g. irradiance level). These impose constraints that must be overcome in attempts to estimate

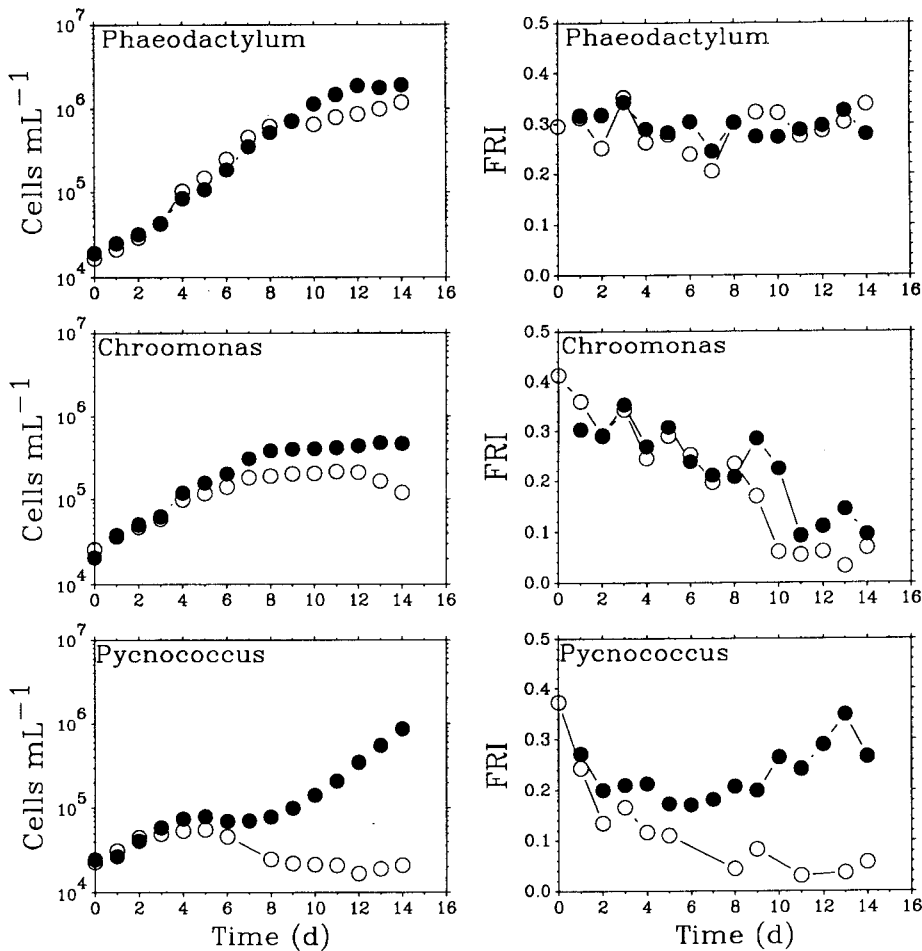


Figure 5. Growth and FRI of *Phaeodactylum tricornutum*, *Chroomonas salina*, and *Pycnococcus provasolii* in unialgal (●) and tri-algal (○) batch cultures.

total primary production of natural assemblages from measurements of FRI for individual species.

There is a second method of estimating photosynthesis from fluorescence which is based on an alternative method of closing the photochemical reaction centres. Instead of using DCMU, it is possible simply to provide enough photons to ensure that all PSII reaction centres will transfer an electron to Q. This is the basis for the "pump-and-probe" technique (Falkowski *et al.*, 1986). In this method, the fluorescence of a weak "probe" flash is measured before (F_p) and after (F_s) a saturating "pump" flash. The greater the capacity for photochemical energy conversion, the larger will be the difference ($F_s - F_p$). The robustness of this method has been verified in both laboratory (Kolber *et al.*, 1988) and field studies (Kolber *et al.*, 1990; Falkowski *et al.*, 1991). Importantly, this method provides estimates that can be used to calculate primary production in absolute units, say mg C mg Chl⁻¹ h⁻¹ (Falkowski *et al.*, 1991). Recently, Olson and Zettler (1991) implemented the pump-and-probe method for FCM analysis of single cells. A modification to their flow cytometer enabled the exciting laser beam to be split into a weak probe beam and a stronger pump beam. Experiments with the diatom *Lauderia borealis* indicated a strong correlation of fluorescence enhancement between FCM-based pump-probe measurements and bulk-based DCMU-addition measurements. In parallel to the problem of detection sensitivity in the FCM-based DCMU method, the weak probe flash used here also imposes a serious constraint on the size of cell that can be measured.

Cell sorting methods

The goal of recovering bulk primary production from single cell analysis is a tantalizing prospect. In this regard, our considerations are directed towards methods capable of apportioning ¹⁴C activity to individual cells after a conventional *in vitro* incubation experiment. There are at least three such methods: microautoradiography (Brock and Brock, 1968; Watt, 1971), isolation by micropipette of single cells visible under a dissecting microscope (Rivkin and Seliger, 1981), and FCM sorting (Li, 1986). Each method has its strengths and weaknesses but it is beyond the scope of this short review to consider them except in the case of FCM sorting.

Particle sorting by FCM is a procedure that yields preparations of cells whose properties (e.g. light scatter, chlorophyll fluorescence, phycoerythrin fluorescence, etc.) are circumscribed by values defined by the researcher. As such, the taxonomic identification of a sorted cell is only as good as resolved by the defining

properties. For example, in open ocean samples, a cell that exhibits significant fluorescence in the phycoerythrin waveband and has light scatter characteristics indicative of a size *ca.* 1 μ m can be regarded as *Synechococcus* sp. with fair certainty. On the other hand, a red-fluorescing 5 μ m cell with no phycoerythrin fluorescence could be a member of many different algal classes. In most cases, the taxonomic identity of sorted cells is revealed only by microscopic examination. It is clear that as the number of FCM-measured properties increases, the level of cell-type discrimination also increases (Olson *et al.*, 1989). In coastal waters where there is often an abundance of non-spherical cells (e.g. diatoms), the use of shape-sensitive parameters (Premaffi *et al.*, 1989; Buonnacorsi and Cunningham, 1990; Cunningham, 1990; Cunningham and Buonnacorsi, 1992) will undoubtedly offer better discrimination. Further, automated pattern-recognition of cytometric signatures (Frankel *et al.*, 1989; Balfoort *et al.*, 1992) will be useful in sorting procedures when performed in real time.

The FCM sorting procedure is physiologically harsh: cells experience physical shear within the flow stream, exposure to intense laser irradiation, and a strong electrostatic charge. In estimating the extent of ¹⁴C uptake by FCM-sorted cells, it is therefore important that the sorting procedure be carried out on cells already labelled with the isotope, so that the incubation assay for photosynthesis is not performed on possibly damaged cells (Rivkin *et al.*, 1986). To have confidence in the sorting procedure, the researcher must be assured of the adequacy of various operational considerations. These include an assurance (i) that a sub-population comprising only a small percentage of the total assemblage can be sorted in a reasonable time (Fig. 6A); (ii) that there is a strict proportionality between the amount of ¹⁴C assayed and the number of cells sorted (Fig. 6B); (iii) that the proportionality in (ii) is not compromised by the presence of other species in the sample (Fig. 6C).

The sorting of ¹⁴C-labelled phytoplankton from natural assemblages can be a daunting and discouraging task (Yentsch and Campbell, 1991). Some of the difficulties are (i) the large dynamic range over which cell properties may span, (ii) the low number at which many species are found in unconcentrated sea water, and (iii) the loss of fixed ¹⁴C and change of fluorescence subsequent to sample preservation. The co-occurrence of phytoplankton in the pico-, nano-, and microplankton size ranges means that even a three or four decade logarithmic scale in the measurements of size and fluorescence may not be sufficient to encompass the entire assemblage. In a study of springtime continental shelf phytoplankton, larger cells were sorted using low instrument gains, while smaller cells were sorted using high gains (Fig. 7). Even so, it is possible that still larger or still

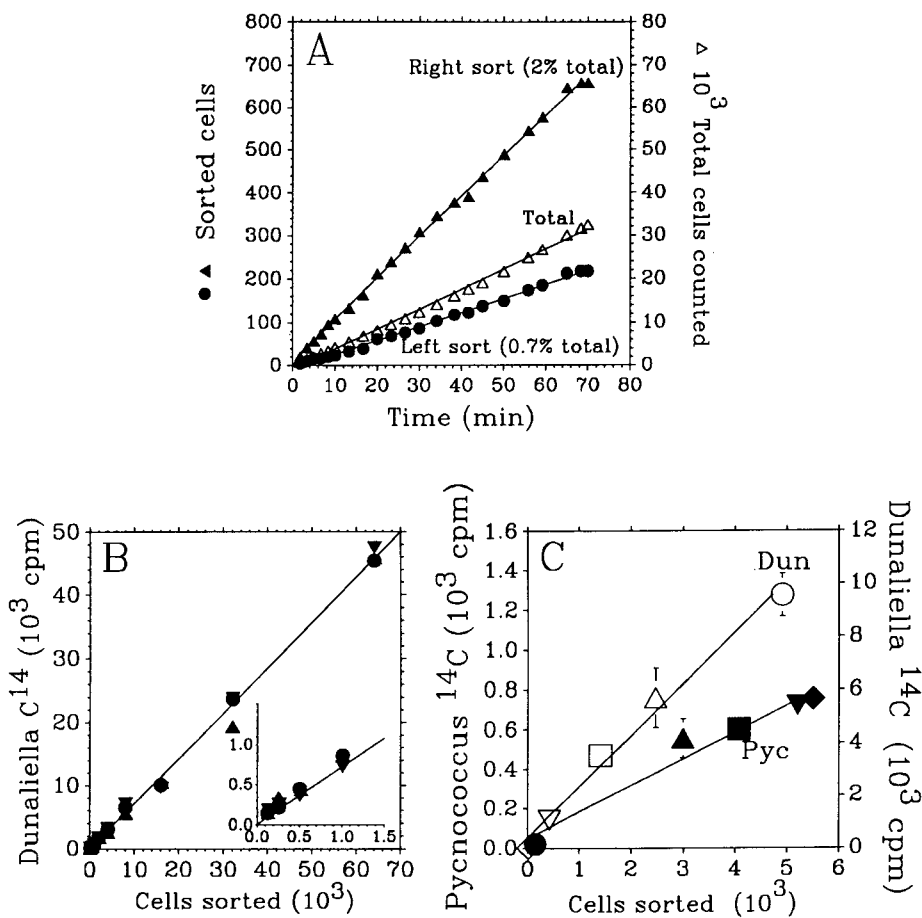


Figure 6. A. Time course of sorting two phytoplankton cell types: the right-sorted population (▲) comprised 2% of the total cells (△) present in the sample; the left-sorted population (●) comprised 0.7% of the total cells. B. Linearity in the amount of ¹⁴C assayed versus the number of *Dunaliella tertiolecta* cells sorted. The inset shows the same data on an expanded scale indicating linearity extending to the origin. C. Sort of two species mixed in five different proportions. Circle symbols refer to unialgal culture of *Dunaliella tertiolecta*; diamond symbols refer to unialgal culture of *Pycnococcus provasolii*; triangles, squares, and inverted triangles refer to bialgal cultures of different proportional composition. Solid symbols refer to *P. provasolii*; hollow symbols refer to *D. tertiolecta*.

smaller cells might have been sorted using other instrument settings. Primary production ($\text{mg C m}^{-3} \text{ h}^{-1}$) is the product of the rate of photosynthesis per cell ($\text{mg C cell}^{-1} \text{ h}^{-1}$) and the cell concentration (cells m^{-3}). The importance of large cells (though few in numbers) is that each individual may be expected to have a high rate of photosynthesis (Goldman, 1988). Vice versa, the importance of small cells (though having low rates of photosynthesis) is that they are often very abundant. The abundance of smaller cells means that it is more feasible to sort a large number for assay of ¹⁴C, hence a lesser variance amongst replicate sorts (groups I-IV, Fig. 7). Conversely, variance amongst replicates was greater for the larger, less numerous cells (groups V and VI, Fig. 7).

A recent report by Casotti *et al.* (1990) described the use of FCM to sort coccolithophores at natural concentrations as low as 25 cells m^{-3} . In this study, the flow cytometer was modified to allow analyses of large volumes of sea water (tens of millilitres) and to measure the polarization of light scattered in the forward direction. The depolarization of forward scattered light by coccoliths was used as a diagnostic feature for the recognition and sorting of coccolithophores.

The problem of preservative-induced changes in fluorescence and ¹⁴C content is a major consideration that requires more study. Aldehydes are commonly used to preserve phytoplankton but they have been shown to change the optical properties (Navaluna *et al.*, 1989) and ¹⁴C retention (Silver and Davoll, 1978) of various

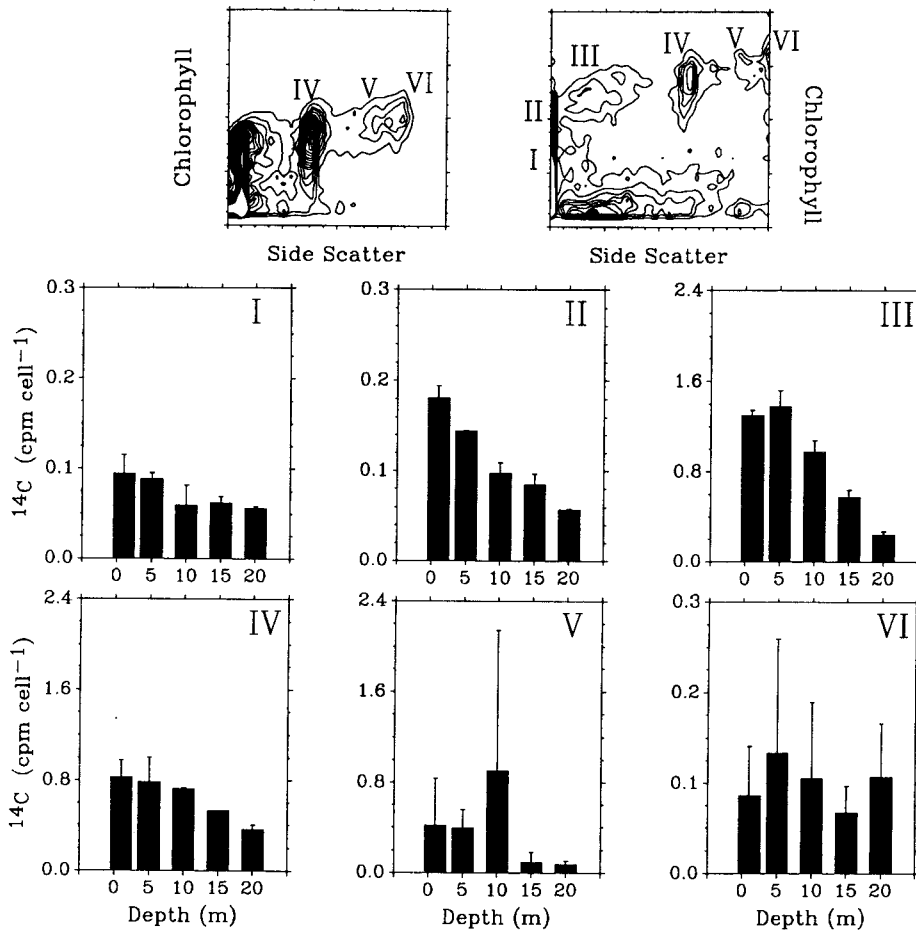


Figure 7. FCM sort of six phytoplankton groups (I–VI) in sea water. Samples were collected from the Roseway Basin (Nova Scotian Shelf; 43°18.06'N 65°10.89'W, 7 April 1991), inoculated with $\text{NaH}^{14}\text{CO}_3$ ($15 \mu\text{Ci ml}^{-1}$) and incubated *in situ* for 12 h. Incubations were terminated by addition of HgCl_2 ($10 \mu\text{M}$) and samples were stored at 4°C until sorted 3–4 weeks later. The upper left panel shows the cytometric signature (Chl fluorescence vs orthogonal light scatter; 3-decade logarithmic scales) at low instrument gains to sort the larger groups IV–VI. The upper right panel shows the signature at high instrument gains to sort the smaller groups I–III. Groups I and II, although indistinguishable by light scatter, were distinctly different on the basis of Chl fluorescence. The six bar histograms plot radioactivity per cell versus water-column depth for each of the phytoplankton groups. Error bars denote variability in duplicate or triplicate sorts of the same preserved sample.

species. For microautoradiography, the loss of fixed ^{14}C in cells can be avoided by not using any chemical preservatives at all; rather, filtered samples are quick-frozen in liquid N_2 and then freeze-dried (Paerl, 1984). Unfortunately, simple quick-freezing of phytoplankton without prior fixation can result in spurious fluorescence signatures (Vaulot and Ning, 1988; Vaulot *et al.*, 1989). The method of quick-freezing after fixation by glutaraldehyde (Vaulot *et al.*, 1989) is a good preservation method for FCM analysis of several species, but likely unsuitable for FCM sorting because of the varying degree to which ^{14}C is lost from different species. The results shown in Figure 7 are compromised to an unknown extent by the possible loss of fixed ^{14}C . Mercuric

chloride, as used in these experiments, results in 10–50% losses of cellular ^{14}C in laboratory tests using cultured algae (unpublished results).

The utility of FCM sorting to estimate group-specific rates of ^{14}C uptake is best seen in cases where samples do not have to be preserved (Rivkin *et al.*, 1986; Olson *et al.*, 1991). In recent preliminary shipboard experiments using a new benchtop flow sorter (FACSort™, Becton Dickinson Immunocytometry Systems), ^{14}C -labelled phytoplankton were sorted live during a trans-Atlantic cruise. In these experiments, total ^{14}C activity was quantitatively partitioned amongst *Prochlorococcus*, *Synechococcus*, and eukaryotic ultraphytoplankters (Li, in preparation). In other words, primary productivity

(P) was successfully calculated on the basis of FCM analysis and sorting.

$$P = \sum N_i \cdot C_i$$

where N_i represents the FCM estimates of abundance for each cell group i (*Prochlorococcus*, *Synechococcus*, and eukaryotic ultraphytoplankters); and C_i represents the values of ^{14}C uptake per cell derived from FCM sorting of unpreserved cells.

Conclusions

The methods outlined in this survey range from rather simple to highly complex procedures. At one end of this range, sea water is sampled from the ocean and phytoplankton abundance is monitored, in "quasi real-time", using commercially available non-sorting flow cytometers. At the other end, the samples are stained, radiolabelled, or otherwise manipulated, incubated, and preserved before being analysed or sorted, sometimes on sophisticated user-customized instruments. Being based on different physiological or ecological principles, the methods do not all yield the same measure of "primary production"; and certainly each method has its own strengths and weaknesses. The important feature shared by each method, and which stands in contrast to conventional bulk oceanographic methods, is that the properties are measured on a cell-by-cell basis.

At present, the goal of estimating the overall rate of primary production from an FCM analysis of constituent properties has been successfully achieved only in certain circumstances. In particular cases, phytoplankton assemblages have a low species diversity and are dominated by numerically abundant forms that have an easily detectable and recognizable cytometric signature: here, the FCM estimates of growth or photosynthesis would be close to estimates derived from conventional bulk methods. However, in the more general case, difficulties arise when species diversity, numerical sparseness, and optical properties are expressed to extents that instrument configuration poses a significant constraint on a complete description of the entire assemblage. Nevertheless, innovative developments in instrument design, novel applications from cytology, molecular biology, ecology, and enhanced data-handling capabilities all indicate a promising future for oceanographic FCM (Olson *et al.*, 1991) that will undoubtedly lead to more detailed information regarding primary production in the sea.

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References

- Antia, A. N., Carpenter, E. J., and Chang, J. 1990. Species-specific phytoplankton growth rates via diel DNA synthesis cycles. III. Accuracy of growth rate measurement in the dinoflagellate *Prorocentrum minimum*. Mar. Ecol. Prog. Ser., 63: 273-279.
- Balfort, H. W., Snoek, J., Smits, J. R. M., Breedveld, L. W., Hofstraat, J. W., and Ringelberg, J. 1992. Automatic identification of algae: neural network analysis of flow cytometric data. J. Plankton Res., 14: 575-589.
- Børsheim, K. Y., Harboe, T., Johnsen, T., Norland, S., and Nygaard, K. 1989. Flow cytometric characterization and enumeration of *Chrysochromulina polylepis* during a bloom along the Norwegian coast. Mar. Ecol. Prog. Ser., 54: 307-309.
- Boucher, N., Vault, D., and Partensky, F. 1991. Flow cytometric determination of phytoplankton DNA in cultures and oceanic populations. Mar. Ecol. Prog. Ser., 71: 75-84.
- Brock, M. L., and Brock, T. D. 1968. The application of microautoradiographic techniques to ecological studies. Mitt. Int. Verein. Limnol., 15: 1-29.
- Buonaccorsi, G., and Cunningham, A. 1990. Azimuthal inhomogeneity in the forward light scattered from microalgal colonies, and its use as a morphological indicator in flow cytometry. Limnol. Oceanogr., 35: 1170-1175.
- Carpenter, E. J., and Lively, J. S. 1980. Review of estimates of algal growth using ^{14}C tracer techniques. In Primary productivity in the sea, pp. 161-178. Ed. by P. G. Falkowski. Plenum Press, New York.
- Casotti, R., Olson, R. J., and Zettler, E. R. 1990. Sea-going flow cytometric discrimination of coccolithophorids. In NATO Advanced Study Institute: individual cell and particle analysis in oceanography. Acquafredda di Maratea, Italy, Oct. 1990. Abstract.
- Chang, J., and Carpenter, E. J. 1988a. Species-specific phytoplankton growth rates via diel DNA synthesis cycles. I. Concept of the method. Mar. Ecol. Prog. Ser., 43: 105-111.
- Chang, J., and Carpenter, E. J. 1988b. Species-specific phytoplankton growth rates via diel DNA synthesis cycles. II. DNA quantification and model verification in the dinoflagellate *Heterocapsa triquetra*. Mar. Ecol. Prog. Ser., 44: 287-296.
- Chang, J., and Carpenter, E. J. 1991. Species-specific phytoplankton growth rates via diel DNA synthesis cycles. V. Application to natural populations in Long Island Sound. Mar. Ecol. Prog. Ser., 78: 115-122.
- Chisholm, S. W., Olson, R. J., and Yentsch, C. M. 1988a. Flow cytometry in oceanography: status and prospects. EOS, 69: 562-572.
- Chisholm, S. W., Olson, R. J., Zettler, E. R., Goericke, R., Waterbury, J. B., and Welschmeyer, N. A. 1988b. A novel free-living prochlorophyte abundant in the oceanic euphotic zone. Nature, 334: 340-343.
- Cucci, T. L., Yentsch, C. M., and Sandvik, R. 1992. PKH-2 as an indicator of phytoplankton division rates: problems with variability. Signal and Noise, 5: 2-3.
- Cullen, J. J., and Renger, E. H. 1979. Continuous measurement of the DCMU-induced fluorescence response of natural phytoplankton populations. Mar. Biol., 53: 13-20.

- Cullen, J. J., Yentsch, C. M., Cucci, T. L., and MacIntyre, H. L. 1988. Autofluorescence and other optical properties as tools in biological oceanography. *In* Ocean optics IX, pp. 149–156. SPIE Vol. 925.
- Cunningham, A. 1990. Fluorescence pulse shape as a morphological indicator in the analysis of colonial microalgae by flow cytometry. *J. Microbiol. Meth.*, 11: 27–36.
- Cunningham, A., and Buonaccorsi, G. A. 1992. Narrow-angle forward light scattering from individual algal cells: implications for size and shape discrimination in flow cytometry. *J. Plankton Res.*, 14: 223–234.
- Demers, S. (ed.). 1991. Particle analysis in oceanography. NATO ASI Series G, Ecological Sciences, Vol. 27. 416 pp. Springer-Verlag, Berlin.
- Dorsey, J., Yentsch, C. M., Mayo, S., and McKenna, C. 1989. Rapid analytical technique for the assessment of cell metabolic activity in marine microalgae. *Cytometry*, 10: 622–628.
- Dubelaar, G. B. J., Groenewegen, A. C., Stokdijk, W., van den Engh, G. J., and Visser, J. W. M. 1989. Optical plankton analyser: a flow cytometer for plankton analysis, II: specifications. *Cytometry*, 10: 529–539.
- Falkowski, P., and Kiefer, D. A. 1985. Chlorophyll a fluorescence in phytoplankton: relationship to photosynthesis and biomass. *J. Plankton Res.*, 7: 715–731.
- Falkowski, P. G., Wyman, K., Ley, A. C., and Mauzerall, D. C. 1986. Relationship of steady-state photosynthesis to fluorescence in eucaryotic algae. *Biochim. Biophys. Acta*, 849: 183–192.
- Falkowski, P. G., Ziemann, D., Kolber, Z., and Bienfang, P. K. 1991. Role of eddy pumping in enhancing primary production in the ocean. *Nature*, 352: 55–58.
- Frankel, D. S., Olson, R. J., Frankel, S. L., and Chisholm, S. W. 1989. Use of a neural net computer system for analysis of flow cytometric data of phytoplankton populations. *Cytometry*, 10: 540–550.
- Furuya, K., and Li, W. K. W. 1992. Evaluation of photosynthetic capacity of phytoplankton by flow cytometric analysis of DCMU-enhanced chlorophyll fluorescence. *Mar. Ecol. Prog. Ser.*, 88: 279–287.
- Gala, W. R., and Giesy, J. P. 1990. Flow cytometric techniques to assess toxicity to algae. *In* Aquatic toxicity and risk assessment: thirteenth volume, pp. 237–246. Ed. by W. G. Landis and W. H. van der Schalie. American Society for Testing and Materials, Philadelphia.
- Goldman, J. C. 1988. Spatial and temporal discontinuities of biological processes in pelagic surface waters. *In* Toward a theory on biological-physical interactions in the world ocean, pp. 273–296. Ed. by B. J. Rothschild. Kluwer Academic, Deventer, The Netherlands.
- Guillard, R. R. L., Keller, M. D., O'Kelly, C. J., and Floyd, G. L. 1991. *Pycnococcus provasolii* gen. et sp. nov., a coccoid prasinocanthin-containing phytoplankton from the western North Atlantic and Gulf of Mexico. *J. Phycol.*, 27: 39–47.
- Horan, P. K., and Slezak, S. E. 1989. Stable cell membrane labelling. *Nature*, 340: 167–168.
- Kolber, Z., Wyman, K. D., and Falkowski, P. G. 1990. Natural variability in photosynthetic energy conversion efficiency: a field study in the Gulf of Maine. *Limnol. Oceanogr.*, 35: 72–79.
- Kolber, Z., Zehr, J., and Falkowski, P. 1988. Effects of growth irradiance and nitrogen limitation on photosynthetic energy conversion in photosystem II. *Plant Physiol.*, 88: 923–929.
- Lande, R. L., Li, W. K. W., Horne, E. P. W., and Wood, A. M. 1989. Phytoplankton growth rates estimated from depth profiles of cell concentration and turbulent diffusion. *Deep-Sea Res.*, 36: 1141–1159.
- Li, W. K. W. 1986. Experimental approaches to field measurements: methods and approaches. *In* Photosynthetic picoplankton, pp. 251–286. Ed. by T. Platt and W. K. W. Li. Can. Bull. Fish. aquat. Sci., 214.
- Li, W. K. W. 1989. Shipboard analytical flow cytometry of oceanic ultraphytoplankton. *Cytometry*, 10: 564–579.
- Li, W. K. W. 1990. Particles in "particle-free" seawater: growth of ultraphytoplankton and implications for dilution experiments. *Can. J. Fish. aquat. Sci.*, 47: 1258–1268.
- Li, W. K. W., Dickie, P. M., Harrison, W. G., and Irwin, B. D. 1993. Biomass and production of bacteria and phytoplankton during the spring bloom in the western North Atlantic ocean. *Deep-Sea Res.*, 40: 307–327.
- Li, W. K. W., Dickie, P. M., Irwin, B. D., and Wood, A. M. 1992a. Biomass of bacteria, cyanobacteria, prochlorophytes and photosynthetic eukaryotes in the Sargasso Sea. *Deep-Sea Res.*, 39: 501–519.
- Li, W. K. W., Lewis, M. R., and Lister, A. 1992b. Flow cytometric detection of prochlorophytes and cyanobacteria in the Gulf of Policastro, Italy. *Arch. Hydrobiol.*, 124: 309–316.
- Li, W. K. W., and Wood, A. M. 1988. Vertical distribution of North Atlantic ultraphytoplankton: analysis by flow cytometry and epifluorescence microscopy. *Deep-Sea Res.*, 35: 1615–1638.
- McDuff, R. E., and Chisholm, S. W. 1982. The calculation of *in situ* growth rates of phytoplankton populations from fractions of cells undergoing mitosis: a clarification. *Limnol. Oceanogr.*, 27: 783–788.
- Melamed, M. R., Lindmo, T., and Mendelsohn, M. L. (eds.) 1990. Flow cytometry and sorting, second edition, 824 pp. Wiley-Liss, New York.
- Mitchison, J. M. 1971. The biology of cell cycle. Cambridge University Press, Cambridge.
- Navaluna, N. A., Perry, M. J., and Talbot, M. C. 1989. The effect of chemical fixation on some optical properties of phytoplankton. *J. Plankton Res.*, 11: 15–24.
- Neale, P. J., Cullen, J. J., and Yentsch, C. M. 1989. Bio-optical inferences from chlorophyll *a* fluorescence: what kind of fluorescence is measured in flow cytometry? *Limnol. Oceanogr.*, 34: 1739–1748.
- Olson, R. J. 1989. Increased sampling rate on an EPICS flow cytometer. *Signal and Noise*, 2: 5.
- Olson, R. J., Chisholm, S. W., Zettler, E. R., and Armbrust, E. V. 1988. Analysis of *Synechococcus* pigment types in the sea using single and dual beam flow cytometry. *Deep-Sea Res.*, 35: 425–440.
- Olson, R. J., Chisholm, S. W., Zettler, E. R., and Armbrust, E. V. 1990a. Pigments, size, and distributions of *Synechococcus* in the North Atlantic and Pacific Oceans. *Limnol. Oceanogr.*, 35: 45–58.
- Olson, R. J., Chisholm, S. W., Zettler, E. R., Altabet, M. A., and Dusenberry, J. A. 1990b. Spatial and temporal distributions of prochlorophyte picoplankton in the North Atlantic Ocean. *Deep-Sea Res.*, 37: 1033–1051.
- Olson, R. J., Frankel, S. L., and Chisholm, S. W. 1983. An inexpensive flow cytometer for the analysis of fluorescence signals in phytoplankton: chlorophyll and DNA distributions. *J. exp. mar. Biol. Ecol.*, 68: 129–144.
- Olson, R. J., Vault, D., and Chisholm, S. W. 1985. Marine phytoplankton distributions measured using shipboard flow cytometry. *Deep-Sea Res.*, 32: 1273–1280.
- Olson, R. J., and Zettler, E. R. 1991. "Pump and probe" fluorescence using a split beam flow cytometer: potential for measurements of photosynthetic energy conversion efficiency in individual phytoplankton cells. Primary productivity and biogeochemical cycles in the sea, 37th Brookhaven Symposium in Biology, NY. Presentation.

- Olson, R. J., Zettler, E. R., and Anderson, O. K. 1989. Discrimination of eukaryotic phytoplankton cell types from light scatter and autofluorescence properties measured by flow cytometry. *Cytometry*, 10: 636-643.
- Olson, R. J., Zettler, E. R., Chisholm, S. W., and Dusenberry, J. A. 1991. Advances in oceanography through flow cytometry. *In* Particle analysis in oceanography, pp. 351-399. Ed. by S. Demers. Springer-Verlag, Berlin, Heidelberg.
- Orellana, M. V., and Perry, M. J. 1992. An immunoprobe to measure Rubisco concentrations and maximal photosynthetic rates of individual phytoplankton cells. *Limnol. Oceanogr.*, 37: 478-490.
- Orellana, M. V., Perry, M. J., and Watson, B. A. 1988. Probes for assessing single-cell primary production: antibodies against ribulose-1,5-bisphosphate carboxylase (RuBP-CASE) and peridinin/chlorophyll a protein (PCP). *In* Immunochemical approaches to coastal, estuarine and oceanographic questions, pp. 243-262. Ed. by C. M. Yentsch, F. C. Mague, and P. K. Horan. Springer-Verlag, Berlin.
- Paerl, H. W. 1984. An evaluation of freeze fixation as a phytoplankton preservation method for microautoradiography. *Limnol. Oceanogr.*, 29: 417-426.
- Platt, T. 1989. Flow cytometry in oceanography. *Cytometry*, 10: 500.
- Premazzi, G., Buonaccorsi, G., and Zilio, P. 1989. Flow cytometry for algal studies. *Water Res.*, 23: 431-442.
- Rivkin, R. B., Phinney, D. A., and Yentsch, C. M. 1986. Effects of flow cytometric analysis and cell sorting on photosynthetic carbon uptake by phytoplankton in cultures and from natural populations. *Appl. Envir. Micro.*, 52: 935-938.
- Rivkin, R. B., and Seliger, H. H. 1981. Liquid scintillation counting for ^{14}C uptake of single algal cells isolated from natural samples. *Limnol. Oceanogr.*, 26: 780-785.
- Samuelsson, G., and Öquist, G. 1977. A method for studying photosynthetic capacities of unicellular algae based on *in vivo* chlorophyll fluorescence. *Physiol. Plant.*, 40: 315-319.
- Silver, M. W., and Davoll, P. J. 1978. Loss of ^{14}C activity after chemical fixation of phytoplankton: error source for autoradiography and other productivity measurements. *Limnol. Oceanogr.*, 23: 362-368.
- Vaulot, D. 1992. Estimate of phytoplankton division rates by the mitotic index method: the f_{max} approach revisited. *Limnol. Oceanogr.*, 37: 644-649.
- Vaulot, D., Courties, C., and Partensky, F. 1989. A simple method to preserve oceanic phytoplankton for flow cytometric analysis. *Cytometry*, 10: 629-635.
- Vaulot, D., and Ning, X. 1988. Abundance and cellular characteristics of marine *Synechococcus* spp. in the dilution zone of the Changjiang (Yangtze River, China). *Cont. Shelf Res.*, 8: 1171-1186.
- Vaulot, D., and Partensky, F. P. 1992. Cell cycle distributions of prochlorophytes in the north western Mediterranean Sea. *Deep-Sea Res.*, 39: 727-742.
- Vaulot, D., Partensky, F., Neveux, J., Mantoura, R. F. C., and Llewellyn, C. A. 1990. Winter presence of prochlorophytes in surface waters of the northwestern Mediterranean Sea. *Limnol. Oceanogr.*, 35: 1156-1164.
- Veldhuis, M. J. W., and Kraay, G. W. 1990. Vertical distribution and pigment composition of a picoplanktonic prochlorophyte in the subtropical North Atlantic: a combined study of HPLC-analysis of pigments and flow cytometry. *Mar. Ecol. Prog. Ser.*, 68: 121-127.
- Vincent, W. F. 1981. Photosynthetic capacity measured by DCMU-induced chlorophyll fluorescence in an oligotrophic lake. *Freshwater Biol.*, 11: 61-78.
- Waterbury, J. B., Watson, S. W., Valois, F. W., and Franks, D. G. 1986. Biological and ecological characterization of the marine unicellular cyanobacterium *Synechococcus*. *In* Photosynthetic picoplankton, pp. 71-120. Ed. by T. Platt and W. K. W. Li. *Can. Bull. Fish. aquat. Sci.*, 214.
- Watt, W. D. 1971. Measuring the primary production rates of individual phytoplankton species in natural mixed populations. *Deep-sea Res.*, 18: 329-339.
- Yentsch, C. M., and Campbell, J. W. 1991. Phytoplankton growth: perspectives gained by flow cytometry. *J. Plankton Res.*, 13(Suppl.): 83-108.
- Yentsch, C. M., and Horan, P. K. (eds.) 1989. *Cytometry in aquatic sciences*. *Cytometry*, 10: 497-669.
- Yentsch, C. M., Horan, P. K., Muirhead, K., Dortch, Q., Haugen, E., Legendre, L., Murphy, L. S., Perry, M. J., Phinney, D. A., Pomponi, S. A., Spinrad, R. W., Wood, M., Yentsch, C. S., and Zahuranec, B. J. 1983. Flow cytometry and cell sorting: a technique for analysis and sorting of aquatic particles. *Limnol. Oceanogr.*, 28: 1275-1280.
- Yunev, O. A., Saldan, N. V., Finenko, Z. Z., Zenin, V. V., and Babich, I. I. 1990. Analysis of Black Sea phytoplankton by flow cytofluorimetry. *Oceanology*, 30: 377-382.