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

Application of flow cytometry in marine phytoplankton research: current applications and future perspectives*

MARCEL J. W. VELDHUIS and GIJSBERT W. KRAAY

Netherlands Institute for Sea Research. P.O.Box 59, 1790 AB Den Burg, Texel, The Netherlands.
E-mail: Veldhuis@NIOZ.nl

SUMMARY: A brief overview is given of current applications of flow cytometry (FCM) in marine phytoplankton research. This paper presents a selection of highlights and various technical and analytical problems we encountered during the past 10 years. In particular, the conversion of the relative values obtained in terms of size and fluorescence applying FCM to quantitative estimates of cell size, pigment concentration, genome size etc., is addressed. The introduction of DNA -cell-cycle analysis made easily assessable by flow cytometry has been of great importance, allowing *in situ* measurement of species specific growth rates. Key questions in ecology such as factors determining the wax and wane of phytoplankton bloom can now be better answered in terms of species specific growth and mortality. Finally, flow cytometry provides detailed information of the physiological status of the individual algal cells. New staining methods enable us to distinguish between viable and non-viable cells and so will help us to elucidate the importance of "automortality" in aquatic ecosystems.

Key words: autofluorescence, automortality, DNA, flow cytometer, growth rate, phytoplankton, viability.

HISTORY

The first and so far best known application of flow cytometry (FCM) in aquatic sciences is in phytoplankton research originally aimed for ametric and ataxonomic purposes (Paau *et al*., 1978; Yentsch *et al*., 1983). Since the early 80's FCM is generally accepted as an analysing technique (see also Burkill, 1987; Yentsch and Pomponi, 1986; Phinney and Cucci, 1989; Burkill and Mantoura, 1990; Olson *et al*., 1991). Despite the fact that the potentials are enormous it is still not a widely spread instrument, nor has its capacity been fully exploited.

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The configuration of most commercially available instruments is based on dealing with particles varying in size between 0.5 and 30 μm and particle numbers ranging from 10^2 to 10^6 per ml, thus covering a major region of particle size distribution in the ocean (Fig. 1). Essentially the optical characteristics of single particles in a solution are measured during their passage through a narrow beam of light (lamp or laser). For this purpose the instrument is equipped with a set of scatter- and fluorescence detectors (mainly photomultipliers). Due to chlorophyll autofluorescence, allowing easy discrimination of the phytoplankton component, the technique has found its way into phytoplankton research, in particular that of picophytoplankton of the open oceans.

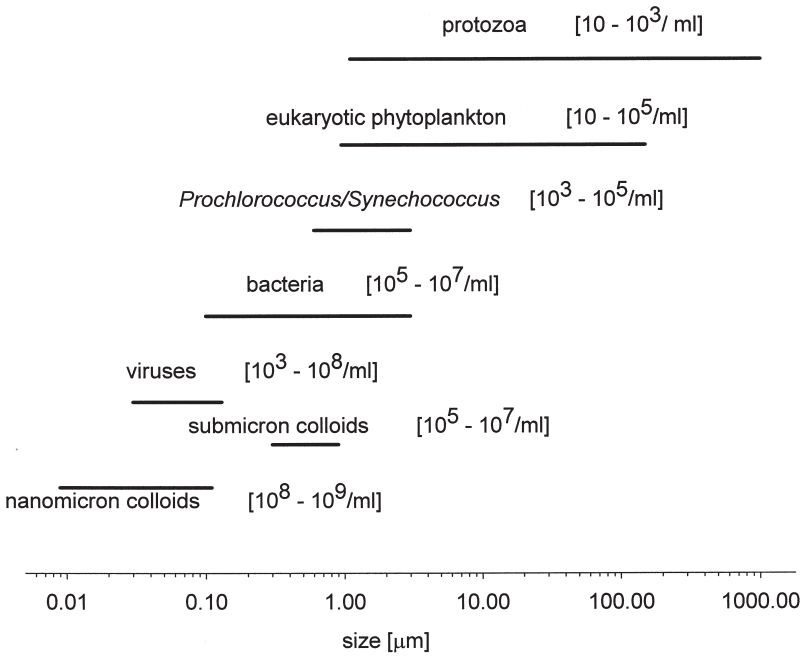

FIG. 1. – Abundances and size class distribution of living and non-living particles in the upper ocean (modified after Koike *et al.*, 1990).

An inventory of unicellular phytoplankton shows that most species fall into the particle range the instrument can detect. However, the variability in size is far larger, ranging over 4 orders of magnitude (0.7 μm to > 1 mm; Fig. 1). In addition, numerous species form chains (in particular diatoms) or produce colonies like *Phaeocystis*. This increases the size range another 10 to 100 fold. In contrast to the smaller species, with numbers exceeding 10^8 per litre, the abundance of the larger phytoplankton species is much lower, often in the order of several hundred cells per litre.

All phytoplankton species contain chlorophyll-*a* as well as DNA and concentrations per cell vary by a factor 50,000 and 20,000, respectively (Burkill and Mantoura, 1990; Holm-Hansen, 1969; Veldhuis *et al.*, 1997a). It is obvious that a single instrument cannot accommodate this dynamic range. To facilitate a broader detection range (> 4 decades) and more detailed morphological features more sophisticated instruments have been designed like the Optical Plankton Analyzer (OPA), its successor EUR-OPA (Peeters *et al.*, 1989; Dubelaar *et al.*, 1989; Peperzak *et al.*, 2000) and more recently its small scale version CytoBuoy (Dubelaar *et al.*, 1999, Dubelaar and Gerritzen, 2000). An alternative instrument to count the larger and more complex particles is FlowCAM (Sieracki *et al.*, 1998). This modified microscope has been designed to visualise digitised images of all particles passing the detector (see also Kachel and Wietzorrek, 2000).

PHYTOPLANKTON (AUTO)FLUORESCENCE

Phytoplankton species contain plant-pigments in a broad variety (Jeffrey *et al.*, 1997), with chlorophyll *a* as the major compound but single source of the red fluorescence signal (emission >610 nm). This chlorophyll fluorescence signal is the principle factor used to discriminate phytoplankton from other particles (Yentsch and Yentsch, 1979). Next, orange fluorescence (emission between 550 to 590 nm) can be used to detect the second group of fluorescing photopigments (phycoerythrin). This photopigment is found only in a limited number of algal groups; typical in many coccoid *Synechococcus* spp. (Wood *et al.*, 1985) and some Cryptophytes. The whole suite of accessory pigments in phytoplankton lack a distinct fluorescence signal. Only in multiple laser instruments additional information can be gained from these pigments (Olson *et al.*, 1988; Hofstraat *et al.*, 1991).

Like chlorophyll-*a*, which is commonly used as a proxy for phytoplankton biomass, the flow cytometric derived *in vivo* autofluorescence signal of the different clusters of phytoplankton can be used to estimate the biomass contribution of each algal group (Li *et al.*, 1993; Shimada *et al.*, 1993). In some cases the FCM-derived fluorescence signal co-varied in a linear manner with the chemically estimated chlorophyll *a* biomass (Veldhuis *et al.*, 1997b). On other occasions both the chlorophyll-*a* concentration and

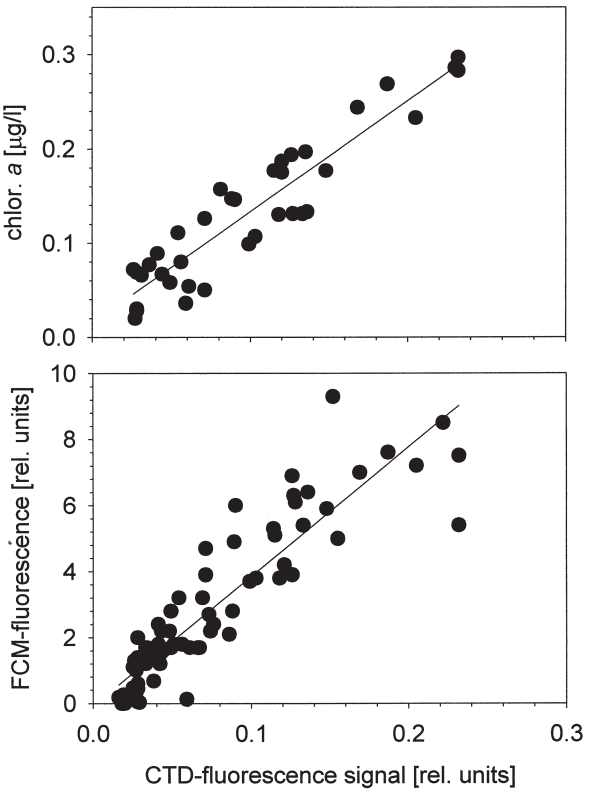

FIG. 2. – *In vivo* -fluorescence of CTD-attached fluorometer versus chlorophyll-*a* concentration (upper panel, n=41, r^2= 0.813) and versus FCM-autofluorescence (lower panel, n=140, r^2=0.881). Samples are taken at 5 locations in the (sub)tropical North Atlantic Ocean.

the FCM-fluorescence signals co-varied with the fluorescence measured with the fluorometer attached to the CTD (oligotrophic North Atlantic Ocean, Fig. 2).

Unfortunately, the chlorophyll fluorescence is also subject to large changes (over one order of magnitude). This is due to a variety of factors like: macro- and micro nutrients as well as the light climate (gradient) and history (diel changes). As a result there is no simple conversion of the chlorophyll based phytoplankton biomass to carbon units.

Alternatively, this rapid adaptation of the fluorescence could also be useful to examine environmentally induced variation in phytoplankton physiology (Sosik *et al.*, 1989; Sakshaug *et al.*, 1987; Veldhuis and Kraay, 1993).

Besides autofluorescence a variety of fluorochromes can be applied for fluorescent labelling cell specific components. Of all applied fluorochromes those labelling the cellular DNA are the most widely used ones in aquatic sciences. Currently a variety of DNA specific dyes are available which can be used to determine the genome size, base pair composition and ploidy (PI, Chrom A3, HOECHST #33342, TOTO, PicoGreen, SYB-Green). The advantage of these dyes is that they are very selective in staining the genome (dsDNA or selective base-pairs) and the recently developed ones do not interfere with the chlorophyll autofluorescence. A detailed study of over 70 different strains of phytoplankton (comprising the major 12 Classes) showed a linear trend between cell size and DNA content (Veldhuis *et al.*, 1997a). This trend was similar to the trend observed for the size and cellular chlorophyll fluorescence (Fig. 3). In both cases there was no Class-related clustering but some grouping was found at the lower range (*Synechococcus* and *Prochlorococcus*) and the higher range (Dinoflagellates) of the size-class spectrum.

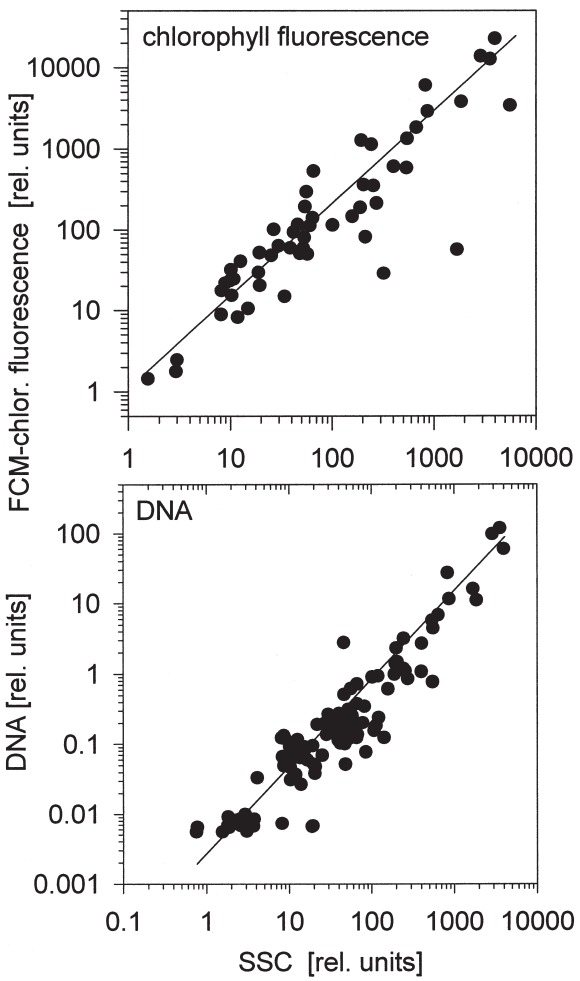

FIG. 3. – Phytoplankton chlorophyll FCM-autofluorescence (>650 nm) versus Side angle Scattered light of 70 strains of phytoplankton (upper panel) and versus DNA content (120 algal strains, lower panel) (modified after Veldhuis *et al.*, 1997a).

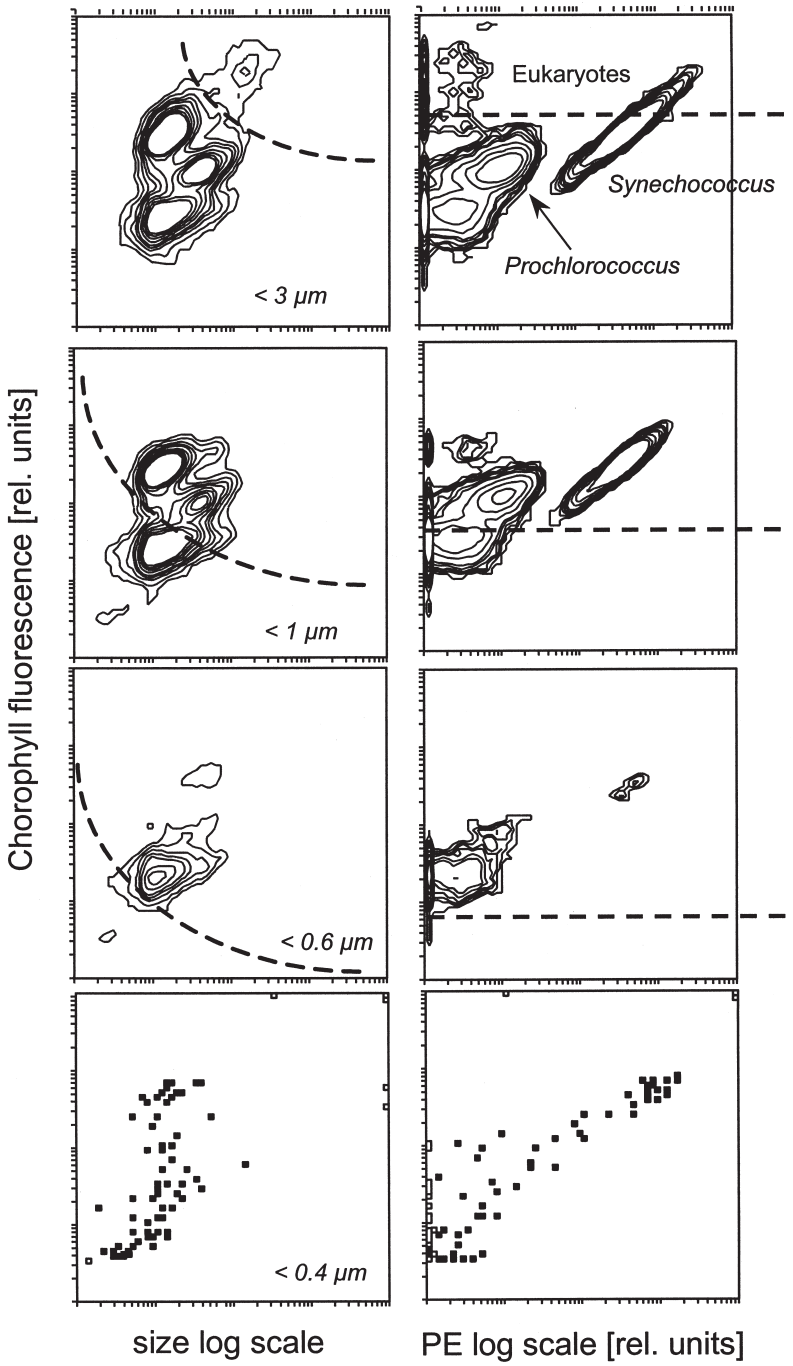

FIG. 4. – Bivariate cell density contour plots showing the side scatter versus chlorophyll and chlorophyll versus PE-fluorescence distribution of oceanic phytoplankton community prefiltered over 3, 1, 0.6 and 0.4 μm filters. Dashed lines indicate cut-off range of the filters (tropical North Atlantic ocean).

On a more detailed level, strain-related differences can be assessed as well (Vaulot *et al*., 1994; Medlin *et al*., 1996; Veldhuis *et al*., 1997a). In *Phaeocystis* and *Emiliania huxleyi* differences in genome size appear to match with differences found in the plant-pigment composition often matching geographical differences (Vaulot *et al*., 1994; Medlin *et al*., 1996).

PHYTOPLANKTON SIZE AND BIOMASS

With respect to the cellular scatter characteristics both Forward angle Scatter (FSC) and Side angle Scatter (SSC) of most phytoplankton species are not very discriminative. Therefore, the usefulness of these signals for taxon specific identification is limited. Only calcifying algae like *Emiliania huxleyi*,

which are densely surrounded by coccoliths (minute plates of calcite) can be distinguished from the other plankton by their typical (high) scatter signature. Despite the fact that the resolution of the scatter signal is enhanced when using a polarising filter set in front of the SSC detector (Olson *et al.*, 1989) this approach has not been widely applied.

For decades phytoplankton biomass has been estimated by converting cell size and shape into cell volume and calculating carbon using empirically derived relationships based on microscopic observations (Strathmann, 1967; Verity *et al.*, 1992). In order to use the flow cytometric data the ametric values of the scatter signals have to be converted to more accurate estimates of cell size or volume. Spherical beads have been used, but these are only reliable in the particle size range above 5 μm (Chisholm, 1992). In general FSC is used as an estimate of cell size mainly because of the good correlation of this parameter with Coulter volume (Olson *et al.*, 1989). For the smaller size range (picophytoplankton, < 3 μm) a different relationship between standard beads and cell sizes is found (Morel, 1991; Charpy and Blanchot, 1998). Alternatively, the plankton community can be size fractionated over filters, which differ in pore size for a rapid, but rather crude determination of the size distribution (Fig. 4).

For taxon specific estimation of the phytoplankton biomass those of *Prochlorococcus* and *Synechococcus* are usually calculated separately because these two species can easily be distinguished from the pico-eukaryotes (Li *et al.*, 1993; Campbell *et al.*, 1994; Li, 1995; Charpy and Blanchot, 1998).

More recently, we applied the genome size to calculate the carbon content of phytoplankton cells. As has been shown earlier (Holm-Hansen, 1969) and recently has been reconfirmed for an even larger size range, the genome size of phytoplankton varies proportionally with the carbon content of cells over 4 orders of magnitude (Veldhuis *et al.*, 1997a). On average the dsDNA in unicellular phytoplankton remains fairly constant and accounts for 2% of the total carbon content. Only dinoflagellates tend to deviate from this relationship with a relatively larger genome size. A comparison of the carbon biomass estimates based on the scatter signals and the DNA-method for tropical phytoplankton (size range of 1 to 7 μm) showed results which are in close agreement (Fig. 5).

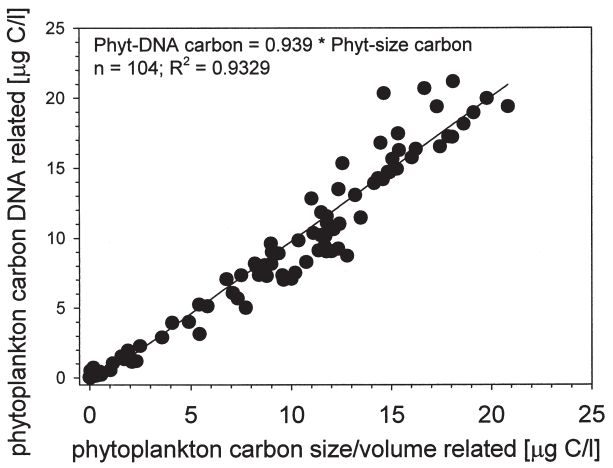

FIG. 5. – Phytoplankton carbon estimated based on size/volume distribution versus carbon based on DNA-carbon relationship as derived in Veldhuis *et al.*, 1997a (samples taken in the tropical North Atlantic).

COUNTING PHYTOPLANKTON CELLS

Since commercial instruments are originally designed for qualitative purposes (semi-) quantitative measurements are still causing major problems. In some instruments this has been solved by counting a fixed volume, e.g. the Coulter XL-MCL is equipped with a 20 μl loop or a module with a fixed counting time corresponding to 1, 10 or 100 μl analysing volume (Optoflow-MICROCYTE). Often beads are used as an internal standard but this is not recommended for several reasons. Firstly, most beads contain toxic substances (e.g. sodium azide or detergents) which can have a negative effect on living cells. Secondly, beads, in particular the smaller ones, stick easily to the tubing and sample vials. Weighting the sample vials prior to and after counting is still one of the best ways to estimate the particle number per unit of volume although not applicable on board a ship.

To check the counting efficiency during data acquisition a plot of the time versus cells number histogram during acquisition can be of help. Deviation from a straight line does indicate unwanted clogging or settling of the particles.

In general count rates should not exceed 200 to 400 particles per second when counting (sub)micron particles (Fig. 6). The actual cell numbers may be underestimated by as much as 70% at counting rates above 1500 per second and effect the intensity of the scatter and fluorescence signals as well. This unwanted side effect of counting particles has been observed in a variety of instruments (Coulter-XL,

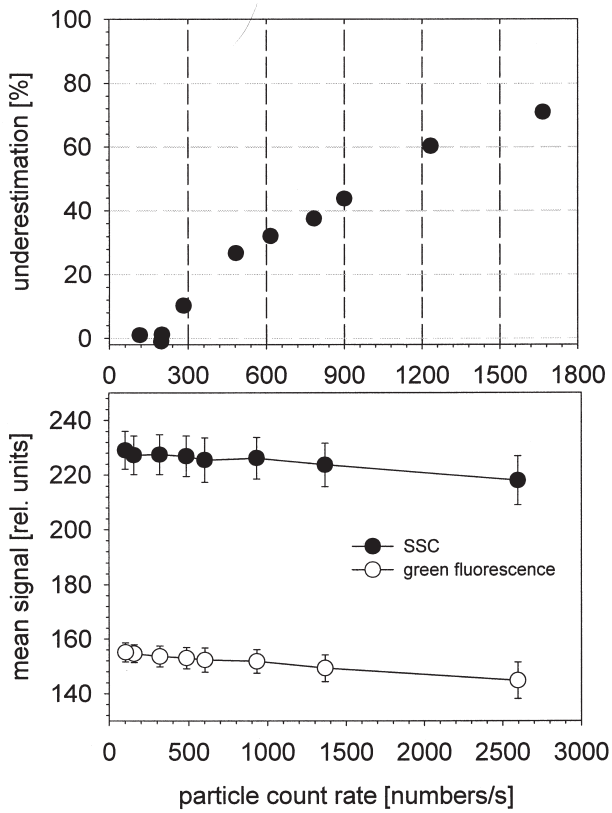

FIG. 6. – Effect of counting efficiency (expressed as % underestimation in particle number) on the counting rate (Coulter XL-MCL, upper panel). Relative variation in scatter and fluorescence signals at different counting rates.

Elite and FACS-Scan). This observation contradicts the theoretical specifications of most modern instruments which are capable of handling much higher count rates, To reduce this effect a proper setting of the laser power, photomultiplier and discriminator/ threshold is crucial.

On the other hand, for proper statistics a sufficient number of cells needs to be analysed. A minimum is 1000 events per run, which implies a c.v. of 10% (Li, 1990). This factor increases in importance in particular in the case of rare cell detection (Leary, 1994) and may come into conflict with the above mentioned count rate problem. Typical conditions at which these conflicting situations occur are: phytoplankton in highly turbid waters or detecting rare organisms in a dense mixture of other organisms.

OCEANIC PHYTOPLANKTON

In particular oceanic research has been benefiting from flow cytometry as a tool to analyse the phytoplankton community. Nowadays, FCM is one of the key instruments in oceanic (pico-) phytoplankton research. Cell abundances, taxonomic diversity and fluorescence signatures can be estimated almost in real time with most commercial instruments (Fig. 7). Moreover, most of these instruments turned out to be stable on board ships.

The number of clusters of phytoplankton to be distinguished in the ocean is often limited (usually not exceeding 8). Except for *Prochlorococcus* and *Synechococcus* the eukaryotes usually show a continuum rather than distinct clusters (Fig. 7). The resolution of this group can be enhanced using special cluster programs (Bakker Schut *et al.*, 1993) or other statistical methods to examine species diversity without (Li, 1997; Carr *et al.*, 1996) or including neural networks (Balvoort *et al.*, 1992; Boddy *et al.*, 1994). Another way to enhance the resolution of the phytoplankton component is to stain the DNA. Since the c.v.'s of the DNA signals is much lower (10 to 15%) than that of scatter or autofluorescence signal (c.v. >50%) nearly identical sized cells appear as separated groups in the DNA frequency histograms (Fig. 8). Nevertheless, to assess the species

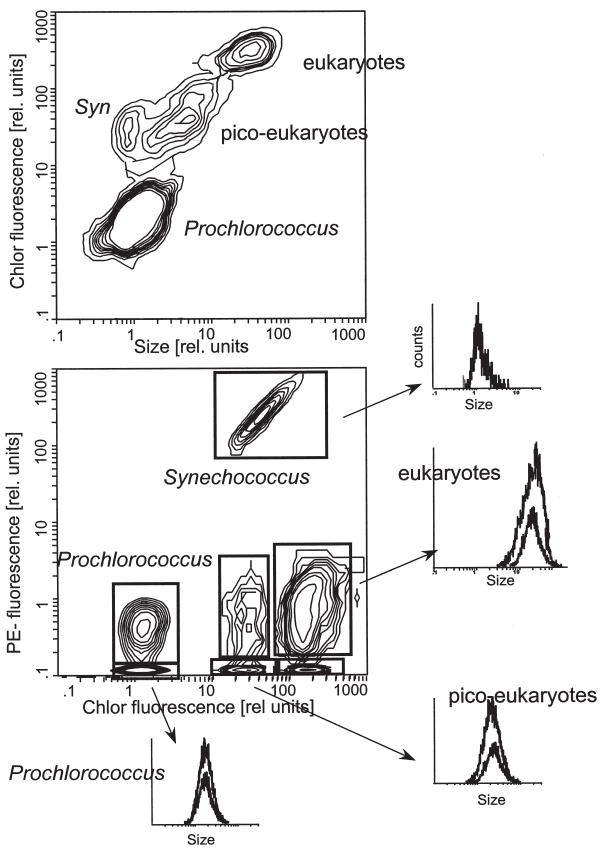

FIG. 7. – Side angle Scattered Signal and autofluorescence signals (chlorophyll, > 650 nm) and phycoerythrin (PE, 530-590 nm) of typical tropical phytoplankton community (North Atlantic, sample taken at 90 m).

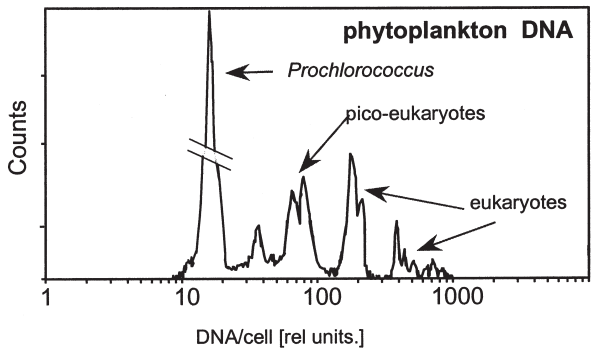

FIG. 8. – Frequency histograms and relative DNA content of oceanic phytoplankton sample showing more details of the diversity in eukaryotic species present (same sample as shown in Fig. 7).

diversity in more detail, FCM measurements need to be complemented with other tools. The combination of cell sorting with (light, TEM, SEM) microscopic observations or immunology and molecular biology (Knauber *et al*., 1996; Urbach and Chisholm, 1998) should provide a detailed description of the species diversity (see Reckermann, 2000).

PROCHLOROCOCCUS; AN EXAMPLE OF A SUCCESS STORY

Flow cytometry, next to plant-pigment analysis, played a prominent role in the 'discovery' of the smallest known phytoplankter so far *Prochlorococcus marinus* (Chisholm *et al*., 1988; Chisholm *et al*., 1992). For excellent reviews on this topic we recommend Partensky *et al*. (1999) and Partensky *et al*. (in press). This unique prokaryotic phytoplankter possesses a unique pigment spectrum including chlorophyll-a_2 and occasionally also chlorophyll-b_2 (Chisholm *et al*., 1988; Partensky *et al*., 1993, Veldhuis and Kraay, 1990). Due to its small size (cell size ranges from 0.6 to 0.9 μm) and dim autofluorescence, the high number (up to 300,000 per ml) were often mistaken for heterotrophic bacteria (Sieracki *et al*., 1995). An inventory of its global distribution reveals that this species is the abundant phytoplankter in all major (sub)tropical oceans both in terms of biomass and productivity: North Atlantic (Neveux *et al*., 1989; Olson *et al*., 1990; Veldhuis

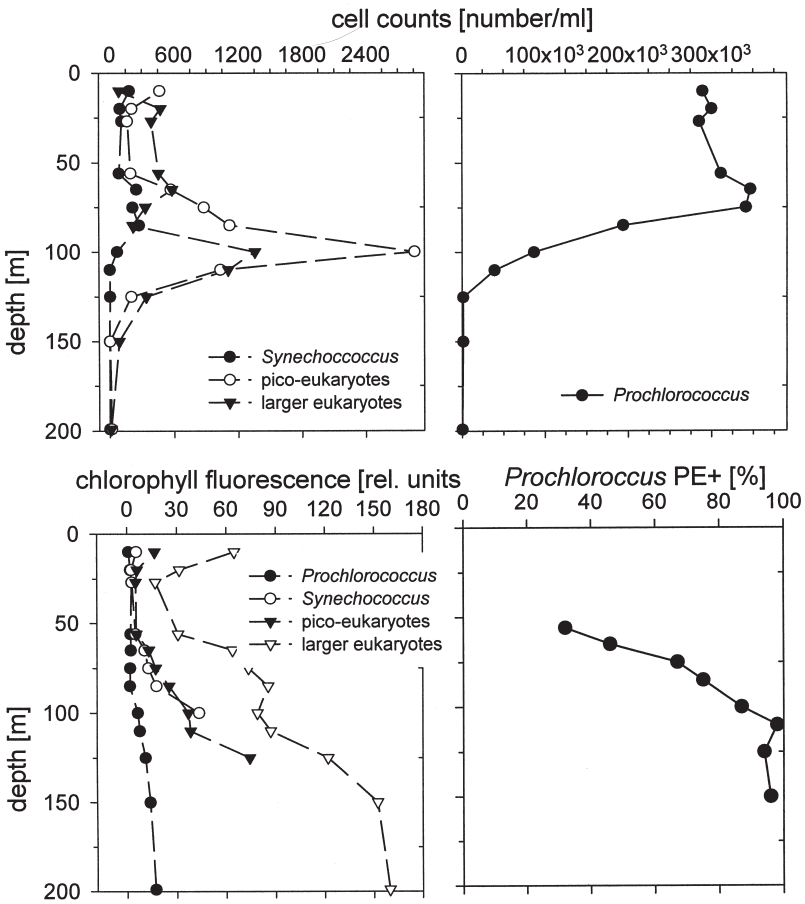

FIG. 9. – Typical vertical profile of phytoplankton abundance, relative cellular fluorescence of different groups identified and % distribution of PE-containing *Prochlorococcus*.

and Kraay, 1990), Pacific (Chavez *et al.*, 1991; Campbell and Vaulot, 1993), Mediterranean (Vaulot *et al.*, 1990; Vaulot and Partensky, 1992), Red Sea and Indian Ocean (Veldhuis and Kraay, 1993; Lindell and Post, 1995). Unlike many other oceanic phytoplankton species *Prochlorococcus* is present over the entire euphotic zone, often below 0.1% of surface irradiance (Fig. 9). Although the species adapts to reduced light intensities by increasing its pigment concentration, strain specific pigment compositions have been recorded (Partensky *et al.*, 1993, 1997; Hess *et al.*, 1996). Whereas the genome size (Veldhuis *et al.*, 1997a), of most isolates examined show great consistency, molecular tools reveal the co-occurrence of different strains (Scanlan *et al.*, 1996). Differences in 16S rRNA sequences are only small (Moore *et al.*, 1998) and strains form major clades (Ferris and Palenik, 1998) but their ecophysiological variability is still considerable.

GROWTH RATE

In ecological studies a proper calculation of the specific growth rates is essential to estimate the role of phytoplankton in the fluxes of energy and matter. Tracing the wax and wane of key phytoplankton species as well as species succession is crucial to the understanding of the functioning of ecosystems. Flow cytometry provides an elegant and accurate method to measure specific cell division rates on the level of individual species. Basically the method is an improvement of the frequency of dividing cells method (Braunwarth and Sommer, 1985; Heller, 1977; Smayda, 1975). The theoretical concepts are well-documented (Chang and Carpenter, 1988; Chang and Carpenter, 1990; Chang and Dam, 1993). Growth rate estimates are based on the DNA frequency distribution traced over a given period of time, usually a 24 h (light/dark) period (Vaulot *et al.*, 1995; Van Bleijswijk and Veldhuis, 1995; Pan and Cembella., 1998; Fig. 10).

Fortunately, the cell division in most phytoplankton species is accompanied by a bimodal DNA distribution with vegetative cells containing exactly 1 or 2 copies of the genome. Using a simple mathematical model, the DNA distribution in the $G_{0/1}$ and G_2 (based on a Gaussian distribution) and an intermediate region, containing cells in the S-phase (Synthesis phase) can be assigned. Next, the cell specific growth rate (μ_{DNA}) is estimated using the fraction of cells in S and G_2 phase during the time course of the measurements (Fig 10). The major advantage of this approach is that growth rates can

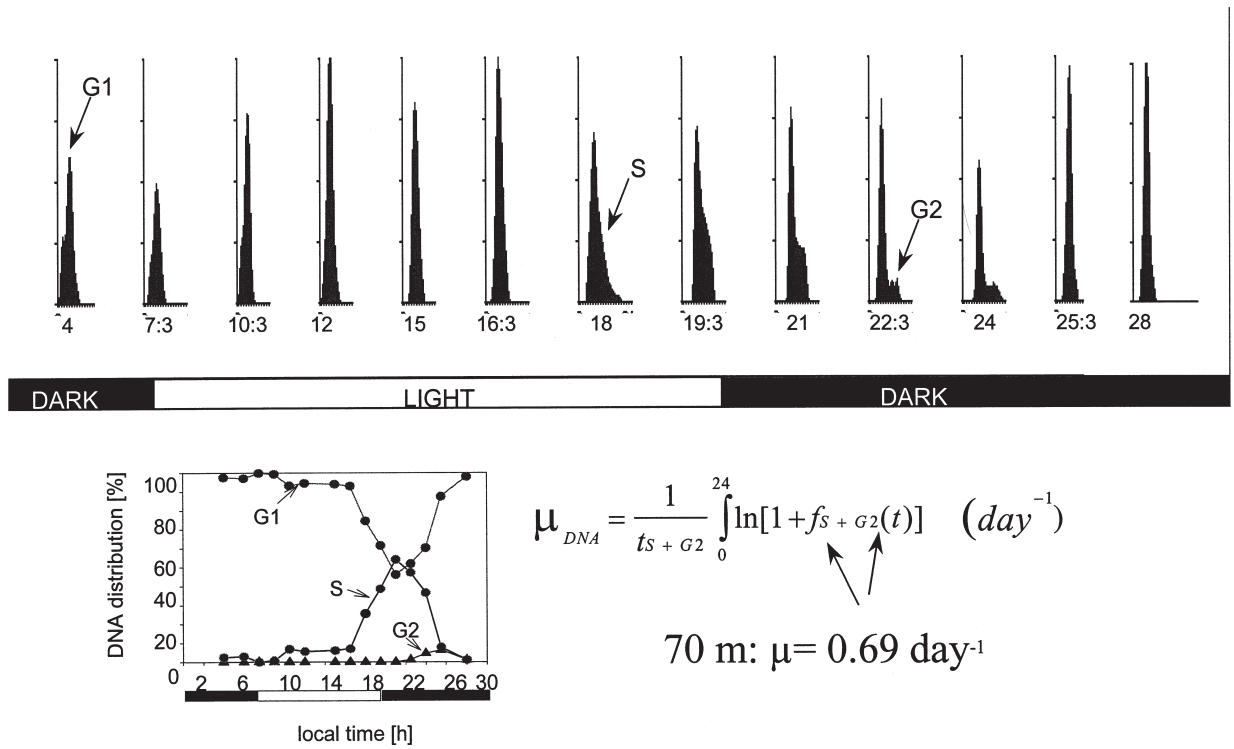

FIG. 10. – DNA frequency histograms of *Prochlorococcus* samples taken at different sampling intervals over a 28-hour (light/dark) period. DNA distribution in $G_{0/1}$, S and G_2 fraction (insert) and equation used to calculated cell specific growth rate (μ_{DNA}). Sample was taken at 70m and calculated growth rate was 0.69 day^{-1}.

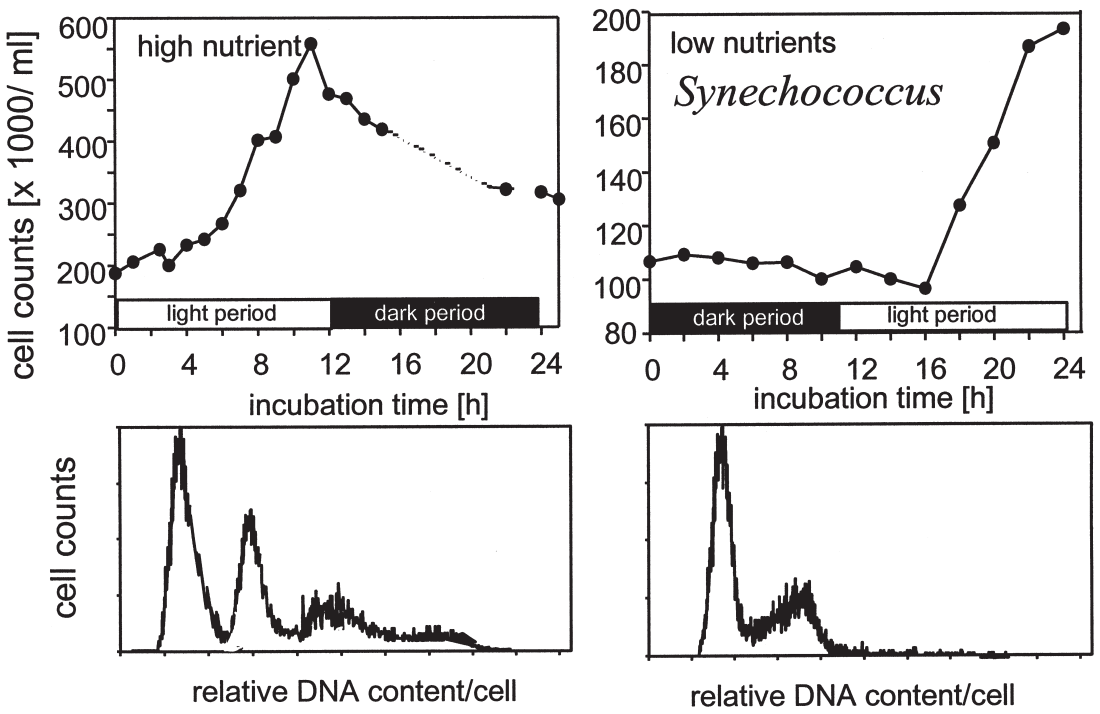

FIG. 11. – Time course changes in cell abundance of two different populations of *Synechococcus* in a nutrient replete (left) and nutrient depleted area (right) Sample collected in the Arabian Sea and incubated in 250 ml bottles (upper panel). Typical DNA distribution with an asynchronized type and bimodal type (lower panel). Samples for DNA profiles are taken shortly before the start of the dark period.

be determined at the level of individual species but most of all *in situ*, omitting any manipulation of the sample.

Recently, the two best studied oceanic phytoplankton genera in this respect, the unicellular *Synechococcus* spp. and *Prochlorococcus* spp. showed a deviation of this bimodal concept. Various strains of the coccoid *Synechococcus* examined show a much more complex cell cycle model matching that of typical prokaryotes (Cooper and Helmstetter or *E. coli* model, Cooper and Helmstetter, 1968). This includes multiple copies of the genome or an asynchrony type with multiple peaks (Armbrust *et al.*, 1989, Binder and Chisholm, 1995, Fig. 11). Also *Prochlorococcus* deviates from this bimodal concept at the higher growth rates with next to a G_2 a clear G_4 phase (Shalapyonok *et al.*, 1998). This atypical DNA frequency distribution in *Synechococcus* complicates an accurate estimate of the DNA distribution in the different stages, hence the calculation of the specific growth rate (Binder and Chisholm, 1995). We observed the asynchronous type in the Arabian Sea under nutrient repleted conditions (Fig. 11), whereas the bimodal type occurred in nutrient deprived waters.

Whereas the DNA-cell-cycle model provides a fairly good estimate of the gross growth rate (μ_G), field populations of phytoplankton are also subject to grazing by microzooplankton and heterotrophic nanoflagellates. Therefore, in any traceable amount of water, the variability in species abundance on a daily base reflects the net change in population size. Combined with the μ_{DNA} the grazing rate can be calculated simultaneously according to:

$$\mu_G = \mu_{DNA} = \mu_{cell} + g \ (d^{-1})$$

Wherein μ_G is gross growth rate, which is equal to μ_{DNA}; μ_{cell} is the overall variation in cell number on a daily base and g is grazing pressure. All parameters can be assessed on the species level or even on level of sub-populations (Reckermann and Veldhuis, 1997).

STAINING AND PROBING APPLICATIONS

Except for staining DNA, very few other aspects of phytoplankton physiology have been addressed although there are numerous ways flow cytometry can be applied in single cell physiology.

Nowadays a variety of FITC-conjugated lectins are commercially available and they have been examined mainly microscopically. These probes

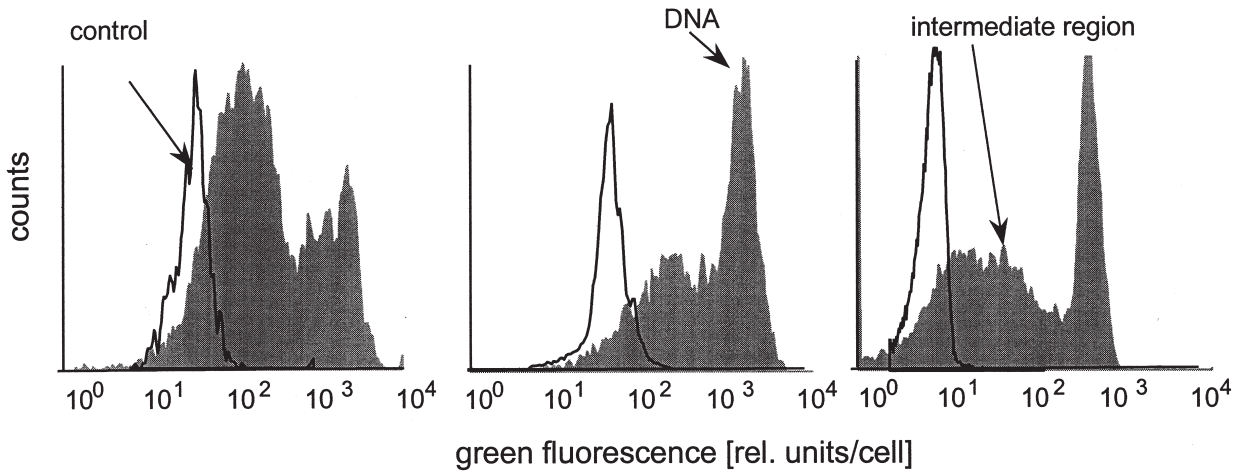

FIG. 12. – Frequency histograms of green fluorescence staining of live phytoplankton samples stained with a DNA specific dye impermeable in live cells. Control: green autofluorescence signal of the cells prior to staining. DNA-signal indicates DNA signal typically found in fixed samples (maximum possible staining). Data of three eukaryotic phytoplankton groups. (North Atlantic in spring 1998). (Veldhuis *et al.*, in press).

were mainly used to study class and cell structure specific binding (Sengbusch *et al.*, 1982; Sengbusch and Müller, 1983; Costas and Rodas, 1994; Costas *et al.*, 1995). Alternative stains to detect specific cellular compounds are Calcofluor White-ST, which stains typically cellulose and chitin fibres, the lipid stains Neutral Red (Crippen, 1974) and Nile Red (Cooksey *et al.*, 1987; Macho *et al.*, 1996). None of these dyes have found a wide application so far in aquatic flow cytometry (see Jochem, 2000).

Next to measuring structural or bio-chemical components FCM can also be applied to address the physiological condition of the phytoplankton cells (Jochem, loc. cit.). In this context phytoplankton viability/automortality is a research topic currently receiving considerable attention. The loss in vitality is nowadays, next to grazing and sedimentation, considered to be the third main factor reducing the size of the population in the field. Cell lysis has been found in particular under poor environmental conditions (Van Bleijswijk *et al.*, 1994; Brussaard *et al.*, 1995; Agustí *et al.*, 1998). It is highly likely, but so far not proven, that this cell death could be based on a programmed process like in multicellular organisms (Brusch *et al.*, 1990). Both light and nitrogen stress have been assigned to induce changes in the protein composition and enhanced activity of proteases in phytoplankton (Berges and Falkowski, 1998). So far, this automortality process has been examined using large sample values. Recently, we have examined an alternative method to test the viability of phytoplankton on the level of the individual cell using FCM. Essentially, the method is based on the membrane integrity of the cells (Veldhuis *et al.*, in press). Like the dye PI, which is used to assess cell death or often better known as apoptosis in the medical field, we used a green fluorescent DNA-specific dye (SYTOX Green) also impermeable in live cells (Fig. 12). It turns out that healthy intact cells remain unstained and dead cells show a full staining of the cellular DNA content corresponding to the signal found in preserved samples. In freshly (unfixed samples) often a third, intermediate population, was observed of cells with a partly comprised cell membrane. Since the disintegration of the membrane structure is typical of a late stage of cell mortality it is clear that cells which are only partly stained have just entered the final stage of automortality. The presence of a demographic structure in a population may well explain e.g. the high degree of variability in UV-B sensitivity as previously observed in the diatom *Cyclotella* sp. (Buma *et al.*, 1995).

Applying the viability assay using freshly collected water samples of the North Atlantic Ocean in spring showed a great variability in staining among the different algal populations present. The percentage of positively stained cells (indicative of comprised membrane permeability) varied from 5% in *Synechococcus* to 40% in other small eukaryotes (Fig. 13). For a spring situation this percentage is relatively high since it indicates the existence of a relatively high proportion of cells with a reduced vitality. To what extent this influences species succession and the cycling of organic matter in the ocean remains a future challenge.

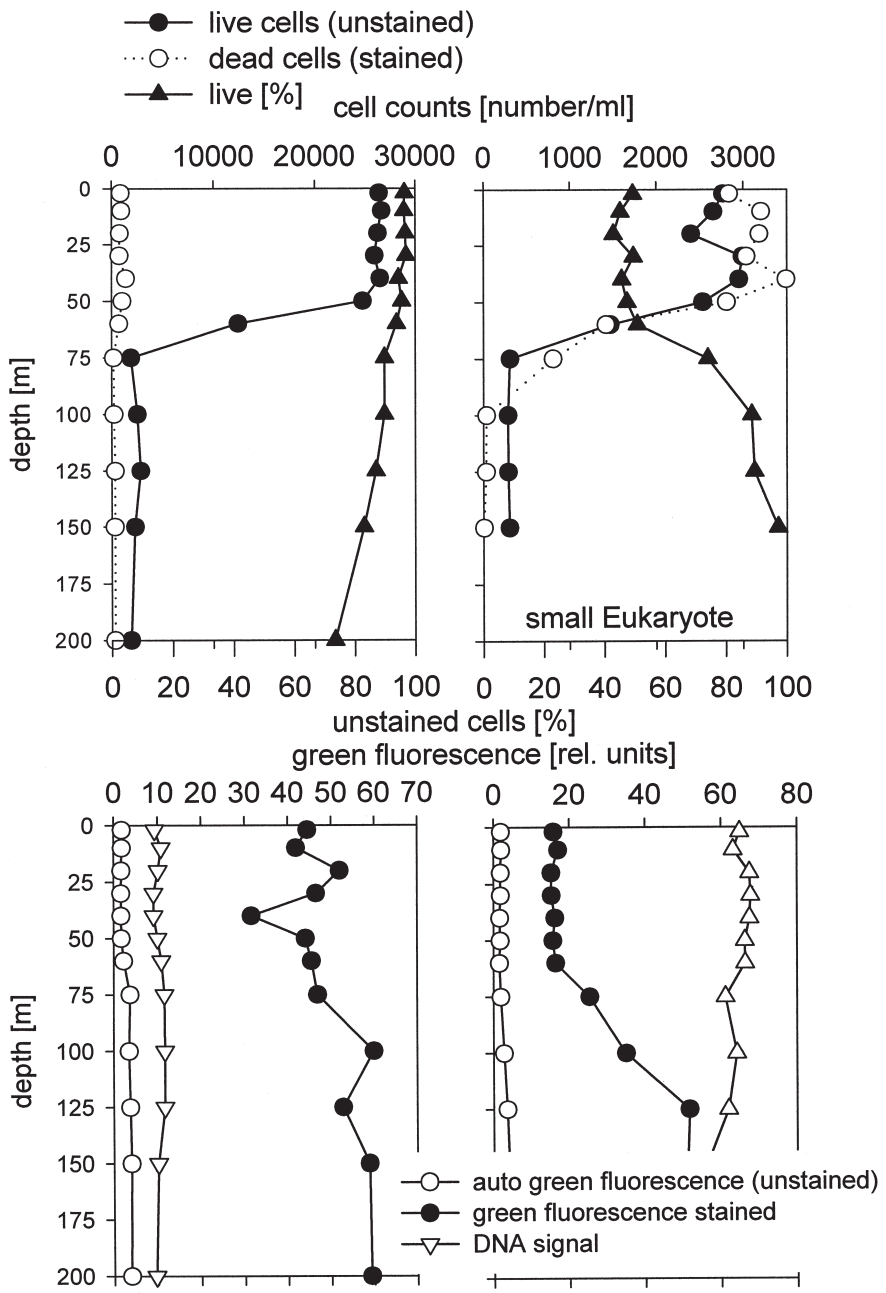

FIG. 13. – Vertical distribution of live and dead cells of *Synechococcus* and a picoeukaryote (North Atlantic in spring 1998, upper panel). Relative green fluorescence of the different live and dead fraction of the population and typical DNA signal in fixed samples (lower panel).

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