Journal of Fluorescence, Vol. 1, No. 4, 1991

Flow Cytometric Discrimination of Phytoplankton Classes by Fluorescence Emission and Excitation Properties

J. W. Hofstraat,[1,2] M. E. J. de Vreeze,[1] W. J. M. van Zeijl,[1] L. Peperzak,[1] J. C. H. Peeters,[1] and H. W. Balfoort[3]

Received November 4, 1991; revised February 21, 1992; accepted March 11, 1992

The ataxonomic discrimination of phytoplankton species on the basis of fluorescence data obtained by multiwavelength excitation in combination with wavelength selective detection in flow cytometry is demonstrated. The discrimination is based on differences in pigment composition between the species, which are reflected in their spectral characteristics. Classification can be done both by making use of the absolute fluorescence intensities and with fluorescence parameter ratios. The latter approach has the advantage that size-related effects and instrument fluctuations are reduced to a large extent. Photoadaptation does influence the absolute as well as the ratioed parameters that are obtained but does not impede the classification into major ataxonomic groups.

KEY WORDS: Flow cytometry; *in vivo* fluorescence spectra; photoadaptation; phytoplankton classification.

INTRODUCTION

In recent years the recognition of the effects of eutrophication of coastal and inland waters has led to an increased need for data on phytoplankton species abundance and distribution. The method used to obtain these data has always been visual analysis by microscope. Of course, this approach offers the most detailed morphological information on the composition of the water samples. On the negative side, cell counting by microscope is time-consuming, not necessarily objective, and less apt to produce statistically sound data. Several methods can be envisaged that do not suffer from the disadvantages of visual analysis. Most methods rely on the automatic registration of characteristic optical properties of phytoplankton. In general, phytoplankton mixtures or cultures are measured in *bulk*. The red fluorescence of the photosynthetic pigment chlorophyll-a, ubiquitous in planktonic algae, is the characteristic feature that is utilized. Equipment that affords the continuous registration of total red fluorescence emission of this pigment has been applied on a routine basis to monitor the phytoplankton biomass, even *in situ* [1–4]. A relatively new development is the application of optical flow cytometers for the analysis of phytoplankton [5,6, and references therein]. In flow cytometry measurements are done on *single* phytoplankton cells.

Flow cytometers allow for the very fast registration of characteristics of individual particles. This is achieved by real-time registration of optical events induced by lasers that are focused on a cuvette through which the particles pass in single file. Flow cytometry has been applied since the 1960s, in particular in biomedical science; to date many instruments are commercially available. In the 1980s applications in marine biology, especially for the detection of small unicellular phytoplankton that is predominant under nutrient-poor con-

[1] Tidal Waters Division, Ministry of Transport and Public Works, P.O. Box 20907, NL-2500 EX The Hague, The Netherlands.
[2] To whom correspondence should be addressed at AKZO Research Laboratories Arnhem, Corporate Research Laboratory, P.O. Box 9300, NL-6800 SB Arnhem, The Netherlands.
[3] Aquatic Ecology Department, University of Amsterdam, Kruislaan 320, NL-1098 SM Amsterdam, The Netherlands.

1053-0509/91/1200-0249$06.50/0 © 1991 Plenum Publishing Corporation

ditions, also have been reported. However, for applications to phytoplankton, which has a tremendous diversity in size and shape, these instruments are not suited [7]. Recently, the construction of a flow cytometer that is suitable for the analysis of such phytoplankton has been reported [8]. The introduction of this "optical plankton analyzer" signifies a major breakthrough in automatic analysis of phytoplankton.

The registration of phytoplankton fluorescence emission and excitation characteristics has already been used as an ataxonomic tool to classify water masses by Yentsch and co-workers and others [9–14]. This was demonstrated by measuring spectral properties of monocultures of different types of phytoplankton. For practical applications one should realize that bulk spectral data are acquired, so that only straightforward data are obtained in exceptional cases, e.g., when one species is predominant in the water mass. On the contrary, in flow cytometry spectral data can be obtained for individual particles [4,15], so that, in principle, classification of phytoplankton species can be done on a cell-by-cell basis. Thus far mainly flow cytometers have been used for measurement of phytoplankton that have been optimized for biomedical applications. Such instruments appear to have limited possibilities for discrimination of phytoplankton species via their fluorescence properties.

In this paper the classification of phytoplankton via wavelength-selective fluorescence emission and excitation measurements is discussed for a flow cytometer that has been especially constructed for the measurement of such particles. Both total fluorescence intensities and fluorescence intensity ratios are applied, both measured for individual particles. It appears that the latter approach, in particular, allows for the straightforward analysis of main phytoplankton groups. The former approach can be used to identify different dominant clusters of particles in the sample, but microscopic observation (preferentially in combination with a cell sorter to select the cells of interest for microscopic inspection) is needed for identification.

Below, bulk fluorescence emission and excitation spectra are presented for a number of algal species in order to define the most optimal excitation and emission wavelengths for flow cytometric analysis. The phytoplankton cultures that are used are representative for important classes of marine and fresh water algae. Subsequently, these cultures are measured with the optical plankton analyzer. The influence of photoinduced effects is examined, as they may strongly affect the spectral properties of the phytoplankton [14]. Finally, results obtained for real samples and mixtures are also presented to demonstrate the feasibility of fluorescence measurement for the discrimination of phytoplankton species.

MATERIALS AND METHODS

Algal Cultures

Monocultures of *Cryptomonas ovata* (CHU-10 medium [16]), *Anabaena flos-aquae* (O_2 medium [17]), *Synechococcus* WH7803, *Rhodomonas* sp., *Dunaliella tertiolecta*, *Isochrysis galbana* (all f/2–Si medium [18]), and *Skeletonema costatum* (f/2 medium [18]) were grown under a 16-h light/8-h dark cycle at 18°C in a Gallenkamp Orbital incubator. Two light intensities were applied, 292 and 45 $\mu Em^{-2}\ s^{-1}$, as determined by a Photodyne 88 XLA with a spherical sensor. Samples were taken from the monocultures the day before the experiment and kept in the dark at a temperature of 4°C. Also, some mixtures of phytoplankton species and of samples from the North Sea and from the freshwater Lake Wolderwijd were examined. These samples were put in brown glass flasks and kept in the dark and on ice until they were measured. To evaluate the effect of photoadaption a culture of *Dunaliella tertiolecta* was grown for several days under seven light intensities (varying from 22.6 to 214.3 $\mu Em^{-2}\ s^{-1}$) in a Gallenkamp Orbital incubator. The intensities were realized by attenuation of the full light of the fluorescent lamps in the incubator; culture flasks were placed in white, diffuse scattering containers, covered with a suitable gray filter to give the required light intensity. Light intensities were measured in the containers with a Photodyne 88 XLA sensor. The growth of the *Dunaliella* cultures at the time of measurement was exponential, as determined by daily flow cytometric cell counts and bulk fluorescence measurements.

Flow Cytometry

A detailed description of the construction of the optical plankton analyzer has been given before [8]. In this study three lasers are used for excitation, at 528 nm (Ar-ion laser at an intensity of about 20 mW at the cuvette), 442 nm (HeCd laser, 25 mW), and 633 nm (HeNe laser, 20 mW). The illuminated area in the cuvette was 5 × 380 μm in the focal plane. It was checked that the excitation intensities used were sufficiently low to prevent any photoinhibitory or photoinduction effects that might result from the interaction of the algae with the consecutive laser beams. Fluorescence emission was

measured in the orange and red wavelength regions. The red (chlorophyll-a) emission induced by the green laser was used to trigger the measurement. The time of flight, the length of the trigger event, was recorded as a measure of the particle length. A survey of the parameters used in this study and their abbreviations is given in Table I.

Triggering is done by setting a threshold on the signal obtained for the FGR parameter. This parameter is chosen for two reasons. First, the Ar-ion laser is the most versatile of the three lasers: its intensity and wavelength (a number of discrete laser emissions from UV to green are available) can be easily varied. Second, only by application of this parameter can trigger cyanobacteria, such as *Synechococcus*, which contain little or no chlorophyll-a, be measured. The phycobiliprotein pigments that are most abundant in these species are not excited in the blue.

To ensure adequate calibration of the flow cytometer, chlorophyll-a-containing standard beads from Flow Cytometry Standards Corporation were added to every sample. These standard beads are most useful because they give measurable signals for all parameters we routinely use for the measurement of phytoplankton. Furthermore, they are easily distinguished from the optical signals of the phytoplankton [19]. For all measurements reported in this paper the mean of the parameters measured for the standard beads in all samples did not differ by more than 5–10% on a logarithmic scale. The coefficient of variance (CV) of the standard beads was 4% for the FGR parameter and 7–10% for the other parameters presented in Table I.

Table I. Survey of the Parameters Used in Flow Cytometric Determinations

Parameter[a]	Angle of detection (degrees)	Laser wavelength (nm)	Detection wavelength (nm)	Property
PLS	90	529	529	Internal structure morphology
FGO	90	529	550–650	Phycobiliproteins
FGR	90	529	>670	Chlorophyll-a, accessory pigments
FBR	90	442	>670	Chlorophyll a
FRR	90	633	>670	Chlorophyll-a and -b, phycocyanin
TOF (for FGR)	90	529	>670	Length

[a]Parameters are, in general, given as logarithmic numbers.

The samples were analyzed at a rate of 20–200 particles per s. Data were transfered from the photodetectors to a Hewlett-Packard 9000-330 minicomputer, which also was used to control the experiment. For each monoculture 4000 eight-parameter logarithmic data (12 bit) were stored. For field samples and mixtures more data were obtained. All measurements were done at least in duplicate. Data files were transfered in listmode to an Apple MacIntosh IICi computer via RedRyder (The Freesoft Company, Beaver Falls, PA). Multiparameter analysis and statistical evaluation were done with DataDesk (Odesta Corp., Northbrook, IL). Averages and intensity ratios were calculated for every phytoplankton species; in addition, the values were examined that were obtained for the chlorophyll standard beads added to all the samples.

Spectrofluorimetry

Fluorescence emission and excitation spectra were recorded on a SPEX Fluorolog 2 spectrofluorimeter (SPEX Industries, Edison, NJ). The fluorimeter is a modular instrument that consists of an ozone-free 450-W xenon arc lamp. 0.22-m double monochromators for selection of excitation and emission light, and a red-sensitive Hamamatsu R928 photomultiplier in a thermoelectrically cooled housing. For correction of the wavelength dependence of the excitation and the emission side of the instrument correction, files are available, in addition to a rhodamine B quantum counter, which monitors directly the intensity of the excitation light. Excitation corrections can be done in the 200- to 600-nm range; emission corrections are available up to 850 nm. To observe any light-induced changes in the spectra, repetitive scans are taken and the effect of prolonged irradiation in the blue on the intensity of the red fluorescence is examined. A more detailed discussion of the measurement and correction procedures is given in Ref. 20. The experiment is controlled by an IBM PC, equipped with dedicated DM3000 software. Spectra were recorded using excitation and emission wavelengths that correspond to the ones used in the flow cytometer. Emission spectra were measured with excitation at 450 and 530 nm. Excitation spectra were obtained while monitoring emission in the orange (at 600 nm) and in the red (at 700 nm).

RESULTS AND DISCUSSION

In this section, first, results of bulk measurements of fluorescence (emission and excitation) of a number

of phytoplankton species from different taxonomic groups are discussed. The species examined are representative for the main classes of phytoplankton that occur in marine and fresh waters. The results are used as an aid in the selection of suitable excitation and emission wavelength regions for the flow cytometric measurements. Second, the flow cytometric fluorescence data obtained for the same cultures (i.e., consisting of only one species) are reported; both absolute intensities and fluorescence intensity ratios have been measured. Next, light-induced effects on the fluorescence measurements are commented upon. Finally, the applicability of algal classification based on measurement of fluorescence properties of individual algae is demonstrated with a few examples.

Spectral Characteristics

Two approaches can be followed to discriminate phytoplankton species on the basis of spectral characteristics. First, the wavelength distribution of the fluorescence emission spectrum can be used. Second, the species-specific differences in the efficiency of excitation of the fluorescence emission can be measured. Figure 1 shows some typical examples of fluorescence emission and excitation spectra that have been obtained for the phytoplankton species used in this study. Table II summarizes the spectral characteristics and the pigment composition of some phytoplankton species representative of classes of algae common in marine and fresh waters. The abbreviations used for the parameters are given in Table I.

The emission spectra of phytoplankton, in general, show the red fluorescence of the photosynthetic pigment chlorophyll-a, which peaks at about 685 nm. In addition, some phytoplankton species (in particular, the cryptophytes and the cyanobacteria) have fluorescent accessory pigments, the phycobiliproteins, which emit in the orange and red regions of the spectrum. Depending on the dominating phycobiliprotein the emission can peak in the 560 to 590-nm range (phycoerythrin PE 545, as in *Rhodomonas* sp., at an emission maximum of 588 nm and in *Synechococcus* WH7803 at an emission maximum of 565) or at 620–650 nm (phycoerythrin PE 565, as in *Cryptomonas ovata*, at an emission maximum of 622 nm and phycocyanin in *Anabaena flos-aquae* at an emission maximum of 652 nm). Orange–red emission therefore is highly selective for the detection of phycobiliprotein containing phytoplankton. The cyanobacterium *Synechococcus* WH 7803 used in this study does not show chlorophyll fluorescence. In emission only phycoerythrin and phycocyanin bands are observed: the 565-nm PE 545 band mentioned before, a phycocyanin band at 651 nm, and an allophycocyanin band peaking at 675 nm. Also, the excitation spectrum recorded by monitoring emission at 720 nm shows only the strong PE 545-nm absorption (maximum at 543 nm).

The other approach is to monitor the intensity dependence of the red and/or orange fluorescence as a function of excitation wavelength. Wavelength dependence of the excitation spectrum also reflects the activity of pigments that do not yield fluorescence emission light but transfer their absorbed energy efficiently to the photosynthetic pigments, in particular, to chlorophyll-a. All phytoplankton species contain such accessory pigments that absorb in characteristic wavelength regions. Chlorophyll-a is excited mainly in the blue (425-nm) and red (660-nm) regions of the spectrum. Chlorophyll-b, the main accessory pigment of chlorophytes and prasinophytes, absorbs in the blue–green (460- to 490-nm) and in the red (610- to 640-nm) region. Chlorophyll-c peaks at about 470 nm, as do the xanthophylls. Bacillariophytes and dinophytes, which are among the most common phytoplankton species in coastal seawater, have fucoxanthin and peridinin, respectively, as main accessory pigments. Both these pigments show increased absorption in the green part of the spectrum, from 500 to 550 nm. Hence, a rough distinction of the phytoplankton species can be made by measuring differences in chlorophyll-a fluorescence intensity as induced by lasers with distinct excitation wavelengths. The orange (phycobiliprotein) fluorescence is not excited in the blue region; the most efficient excitation is in the green/orange, 530- to 600-nm, part of the spectrum.

Based on these bulk measurements three excitation wavelengths were selected in order to achieve maximum discrimination among the main phytoplankton classes: blue excitation at 442 nm, i.e., in the chlorophyll absorption maximum; green excitation at 529 nm, i.e., optimal excitation for bacillariophytes and dinophytes; and red excitation at 633 mm, i.e., optimal excitation for green algae, such as chlorophytes and prasinophytes, and many blue–green algae. Emission is measured in the red (chlorophyll-a) and in the orange (cryptophytes, cyanobacteria) part of the spectrum. To be able to assess the effect of photoinduced effects, flow cytometric measurements were done on cultures incubated at "high" and "low" light intensity.

Photoadaptation Effects

Photoadaptative effects have been well documented in the literature [12, 21, and references therein]. In an attempt to make optimal use of the available light field,

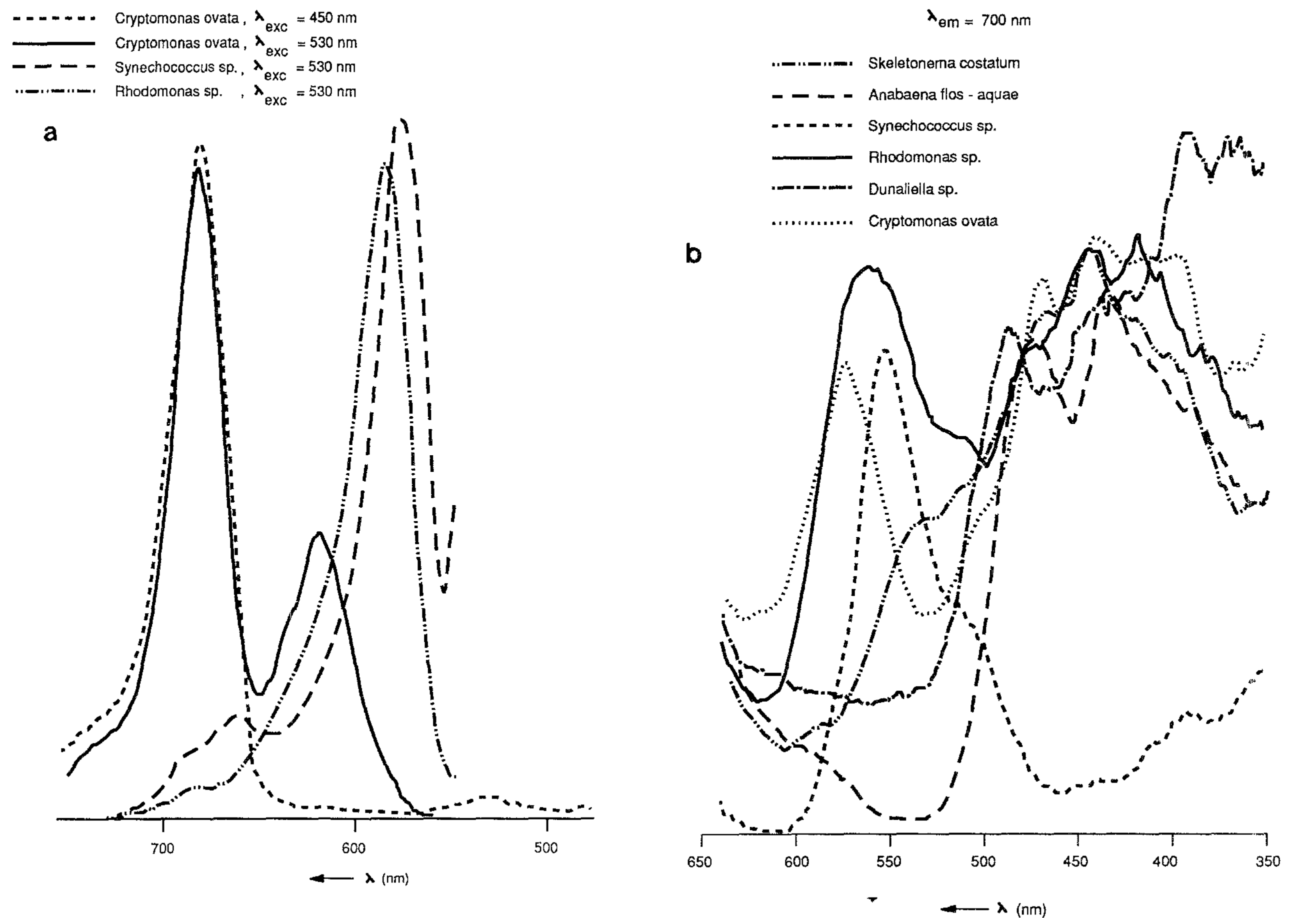

Fig. 1. Fluorescence emission and excitation spectra of some representative algae. Spectra have been totally corrected for the wavelength dependence of the instrument. (a) Emission spectra of *Cryptomonas, Rhodomonas,* and *Synechococcus*, all phycobiliprotein-containing algae. Also, the effect of the excitation wavelength is demonstrated: when excited at 450 nm *Cryptomonas* exclusively shows red chlorophyll fluorescence. In the blue the phycobiliprotein pigments are not excited. (b) Excitation spectra of *Skeletonema, Anabaena, Synechococcus, Rhodomonas, Dunaliella,* and *Cryptomonas*, monitored in the red fluorescence region (at 700 nm). Of particular interest are the blue region, where most algae absorb strongly, and the green region, where the most striking differences in excitation are observed. In the blue region strong absorption by chlorophyll occurs; all algae, apart from *Synechococcus*, contain this pigment. *Synechococcus* shows hardly any absorption in the blue but is strongly excited at 560 nm. The emission observed at 700 nm is probably due mostly to allophycocyanin. In the green the differences are most pronounced: *Anabaena* and *Dunaliella* are not excited very efficiently; the phycoerythrin-containing algae *Synechococcus, Rhodomonas*, and *Cryptomonas* and the fucoxanthin-containing *Skeletonema* are excited much more efficiently. The latter two groups can be discriminated on the basis of their emission properties.

the phytoplankton adapts its photosynthetic system. The adaptation in general is done by influencing the synthesis of pigments and by influencing the efficiency of energy transfer from the accessory pigments to chlorophyll-a. Both processes lead to significant changes in the characteristics of phytoplankton absorption (and excitation) and emission spectra. In particular, the light history of the sample therefore affects the pigment composition: phytoplankton grown under low light conditions has more chlorophyll-a per cell and may have an increased amount of accessory pigments and/or a more efficient energy transfer of the accessory pigments to chlorophyll-a [21]. The effect of light history on the fluorescence emission and excitation spectra was therefore also considered in this study. Spectra as well as flow cytometric characteristics were investigated for samples that had been cultivated at high and low light intensity. Prior to measurement, samples were dark adapted for at least 1 h to remove any transient photoinduced effects.

An example of the effect of photoadaptation is cleary visible in the *Anabaena flos-aquae* emission spectra shown in Fig. 2. The spectrum obtained for *Anabaena* grown under low light conditions shows a much more pronounced phycocyanin band (at about 650 nm) than that

Table II. Spectral Characteristics and Pigment Composition of Some Phytoplankton Species

Species	Taxon	Main pigments
Anabaena flos-aquae	Cyanobacterium	Chlorophyll-a, phycocyanin, myxoxanthin
Cryptomonas ovata	Cryptophyte	Chlorophyll-a and -c, phycoerythrin (PE 565), alloxanthin
Dunaliella tertiolecta	Chlorophyte	Chlorophyll-a and -b, lutein
Isochrysis galbana	Haptophyte	Chlorophyll-a and -c, fucoxanthin
Rhodomonas sp.	Cryptophyte	Chlorophyll-a and -c, phycoerythrin (PE 545), alloxanthin
Skeletonema costatum	Bacillariophyte	Chlorophyll-a and -c, fucoxanthin
Synechococcus WH7803	Cyanobacterium	Phycoerythrin (PE 545), phycocyanin, allophycocyanin
Tetraselmis sp.	Prasinophyte	Chlorophyll-a and -b, lutein

for the culture grown under high light conditions. This observation corroborates the increased importance of the accessory pigment for the phytoplankton grown under low light conditions [21]. As the concentration of the algae is unknown in the bulk spectral measurements, the spectral intensities cannot be compared in an absolute sense.

The effect of photoadaptation on flow cytometric measurements is illustrated in Figs. 3 and 4. Figure 3 shows bivariate plots for *Anabaena*, in which intensity values are depicted for FGR and FBR for cultures grown under high and low light conditions. For comparison, the position of the chlorophyll standard beads is also indicated in the figure. The algae that have been grown under low light conditions have a much higher efficiency for excitation in the wavelength region where the accessory pigments absorb. As mentioned before, this is due to the higher content of these pigments and a more efficient energy transfer to the photosynthetic pigment chlorophyll-a. In addition, the content of chlorophyll-a will be higher in the low light-adapted algae. In Fig. 3 the increased fluorescence intensities for the samples grown under low light conditions are obvious: the cluster formed by the shade-adapted algae has shifted significantly to higher fluorescence intensity values. This is particularly clear for FGR (take the position of the chlorophyll beads as a reference). The increased content of accessory pigment is obvious when parameter ratios are examined (see below). Photoadaptive effects, as discussed for *Anabaena*, are observed for most phytoplankton species. Two trends are observed: the low light-adapted species show higher absolute fluorescence intensities and a relative reduction of the blue (chlorophyll-a)-induced fluorescence in comparison with the other parameters, indicative of increased excitation efficiency of chlorophyll-a via the accessory pigments. The latter observation is corroborated by the bulk spectral data obtained with the spectrofluorimeter. Intensities per particle, of course, can be obtained only with a technique such as flow cytometry, which probes individual cells.

The effect of photoadaptation is particularly clear from Fig. 4. *Dunaliella teriolecta* cultures were grown for several days under seven light levels (between 22.6 and 214.3 $\mu Em^{-2}\ s^{-1}$) and their red fluorescence was measured by flow cytometry, while they were still growing exponentially.

Figure 4 clearly shows the increase in the chlorophyll fluorescence per particle for the cultures that have been grown under lower light conditions, which is indicative of an increased pigment content in the shade-adapted algae.

Flow Cytometry

Absolute Intensities

Flow cytometric measurements are done on individual particles, so that comparisons of "absolute" light intensities per alga can be made. As many algae can be measured individually, information is also obtained on the biological variability of the optical characteristics and hence, indirectly, on that of pigment content, size, and other physiological characteristics. However, the signal strengths rely in an intricate way on laser excitation power, optimization of the excitation and detection optics, photomultiplier high voltage, and other instrumental parameters. Therefore results obtained for one flow cytometer cannot easily be compared to those from another instrument, and even for the comparison of data measured on the same instrument, extreme care has to be taken to ensure adequate calibration. This implies that the results presented below are indicative of the results of multiwavelength flow cytometry but cannot be transferred directly to other instruments. An important difference between between the optical plankton analyzer and most instruments is that in the latter, generally peak maxima ("pulse heights") are used as observables, whereas in the former, the total integrated intensity is obtained. This aspect makes the optical plankton analyzer suitable for measurement of whole particle characteristics, even for larger algae or algae that consist of more than one cell. On the other hand, the integration approach also presents some problems, mainly

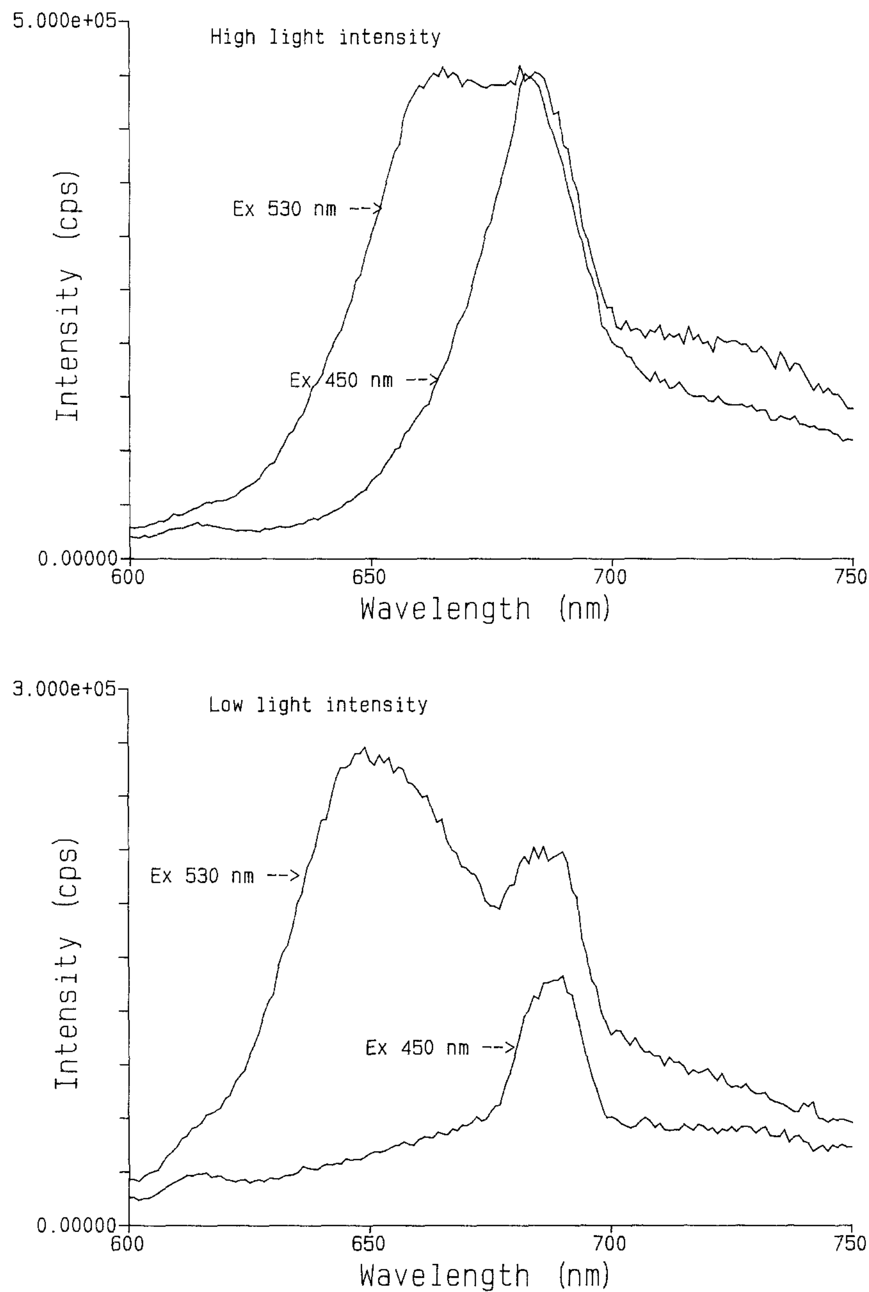

Fig. 2. Fluorescence emission spectra of *Anabaena flos-aquae* cultures grown under high and low light intensity. Note the remarkable changes in the spectrum under excitation at 530 nm. The spectrum is hardly changed when blue excitation is used. Also note that the intensity ratio for red fluorescence as induced in the green and in the blue differs by about a factor of 2 for the low light- and high light-adapted cultures.

in relation to increased dark counts and more difficult calibration of the instrument (e.g., changes in the speed of the particles in the cuvette will lead to changes in the measured intensity value). The latter problem can be mastered by careful calibration; chlorophyll standard beads have been added to every sample to ensure the comparability of all the measurements done on the optical plankton analyzer. In this section the application of absolute parameter intensities for distinction of phytoplankton species is discussed.

In Table III the averaged absolute intensities of the fluorescence parameters, as well as the perpendicular

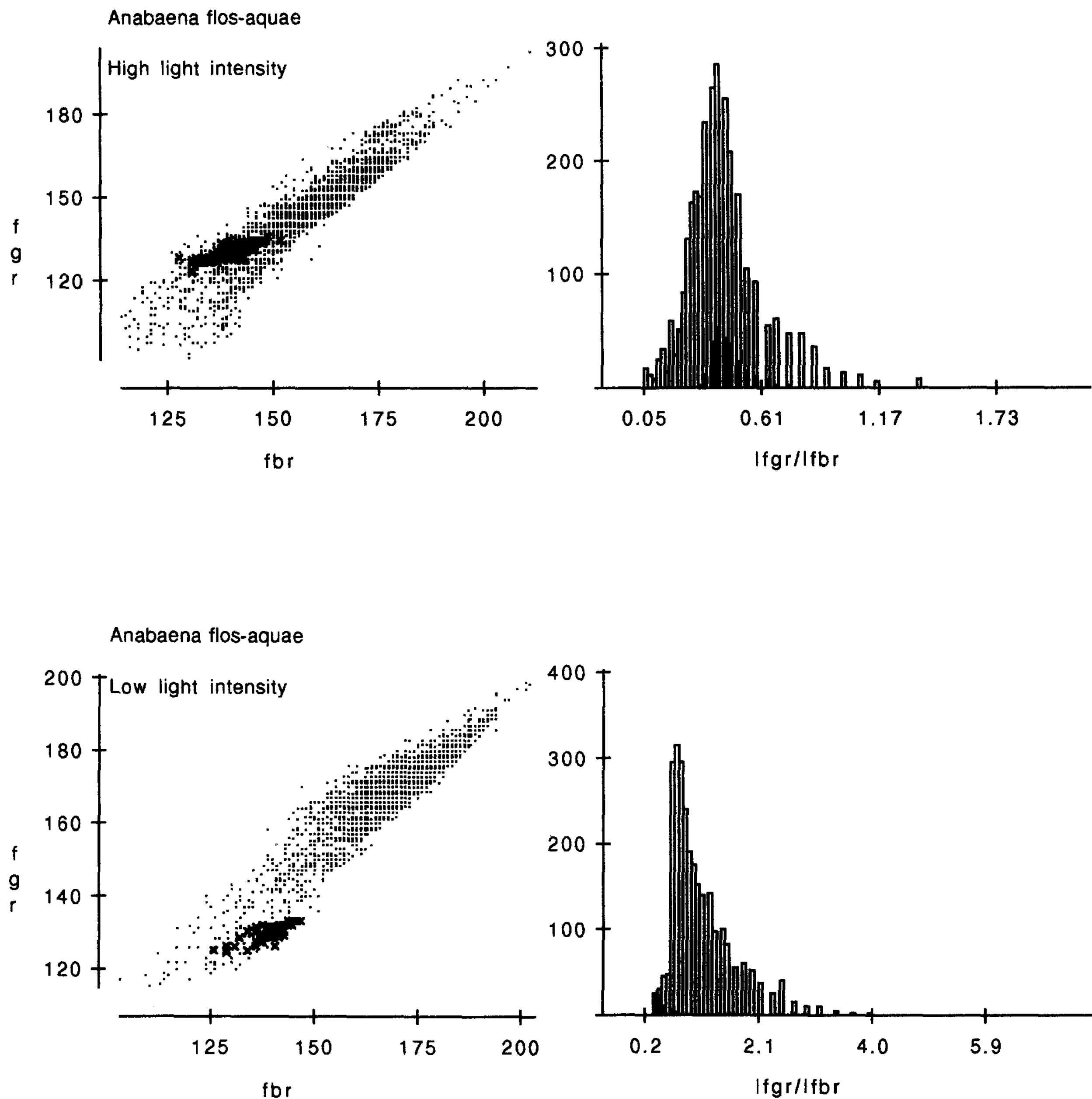

Fig. 3. Flow cytometric data for high light- and low light-adapted *Anabaena flos-aquae*. Also indicated (in boldface) are the chlorophyll standard beads that have been added to the sample for calibration purposes. The low light-adapted culture shows a shift of the *Anabaena* cluster to higher fluorescence values. At the same time the FGR/FBR parameter ratio changes from 0.4 in the high light-adapted culture to 1.1 in the low light-adapted one. For comparison, the chlorophyll standard beads have an average FGR/FBR of about 0.4.

light scatter (PLS) and the time of flight (TOF), are given as measured for pure cultures of the phytoplankton species given in Table II. The parameter values, which are transformed into logarithmic values during the measurement and are stored as such in list mode in the computer, have been manipulated into a linear presentation. The wavelength regions used for the fluorescence parameters have been selected on the basis of the spectral data as discussed in the previous section and are given in Table I. The intensity values are the average of measurements on at least 2000 individuals and have been obtained in at least duplicate experiments. The differ-

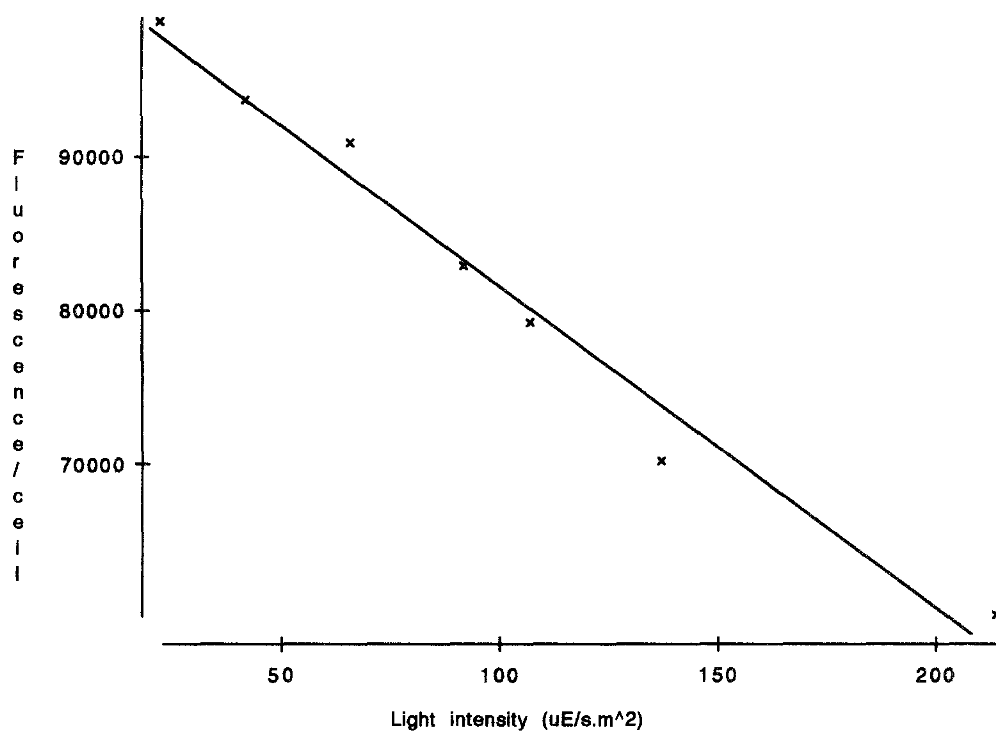

Fig. 4. Photoadaptation effects in the flow cytometric data obtained for *Dunaliella* cultures grown under light intensities varying from 22.6 to 214.3 $\mu Em^{-2} s^{-1}$.

Table III. Intensities of Flow Cytometer Parameters, Transformed to Linear Scale[a] (Values Are the Average of at Least 4000 Observations from Duplicate Experiments)

Species	Light field	FGR (×10⁵)	FGO (×10⁵)	FBR (×10⁵)	FRR (×10⁵)	PLS (×10⁵)	TOF[b]
Anabaena	High	1.4±2.0	0.20±0.45	3.3±3.8	3.5±4.8	2.7±4.9	65±52
	Low	5.2±4.0	1.1±1.0	5.4±4.8	8.4±7.5	3.9±3.2	86±56
Cryptomonas	High	2.4±1.5	2.4±1.6	5.2±2.9	0.8±0.5	3.0±1.6	17±6
	Low	6.4±2.9	7.3±3.6	9.7±4.4	2.0±0.9	3.0±1.6	26±11
Dunaliella	High	0.22±0.06	0.005±0.002	0.42±0.14	0.15±0.04	0.87±0.32	4.2±1.5
	Low	0.63±0.18	0.007±0.002	0.89±0.36	0.43±0.12	0.80±0.34	8.5±2.3
Isochrysis	High	0.14±0.06	0.004±0.001	0.3±0.15	0.05±0.02	0.15±0.09	3±2
	Low	0.37±0.14	0.005±0.002	0.45±0.19	0.11±0.04	0.13±0.09	7±3
Rhodomonas	High	1.4±0.4	16±4	1.3±0.3	0.16±0.04	0.64±0.25	10±3
	Low	2.0±0.5	19±4	1.8±0.5	0.32±0.08	0.51±0.35	14±3
Skeletonema	High	0.71±0.81	0.009±0.009	1.1±1.2	0.24±0.22	0.88±0.99	14±17
	Low	2.2±1.4	0.02±0.01	2.0±1.3	0.51±0.31	1.7±1.1	35±18
Synechococcus	High	0.04±0.03	0.16±0.11	0.007±0.001	0.007±0.001	0.015±0.03	−1±1
	Low	0.04±0.03	0.40±0.30	0.01±0.04	0.009±0.002	0.017±0.02	−1±1

[a]Parameters are transformed to logarithmic values (scaled to 8-bit numbers) during the measurement. Transformation to linear values requires manipulation of the list mode data that have been stored.

[b]The TOF value is measured on-line as the time the FGR signal is above threshold and transformed to a logarithmic value. The TOF value in this table has been transformed to a linear value and corrected for the velocity of the particles in the cuvette and for the area illuminated by the laser. The TOF values, therefore, should be representative for the particle length, expressed in µm..

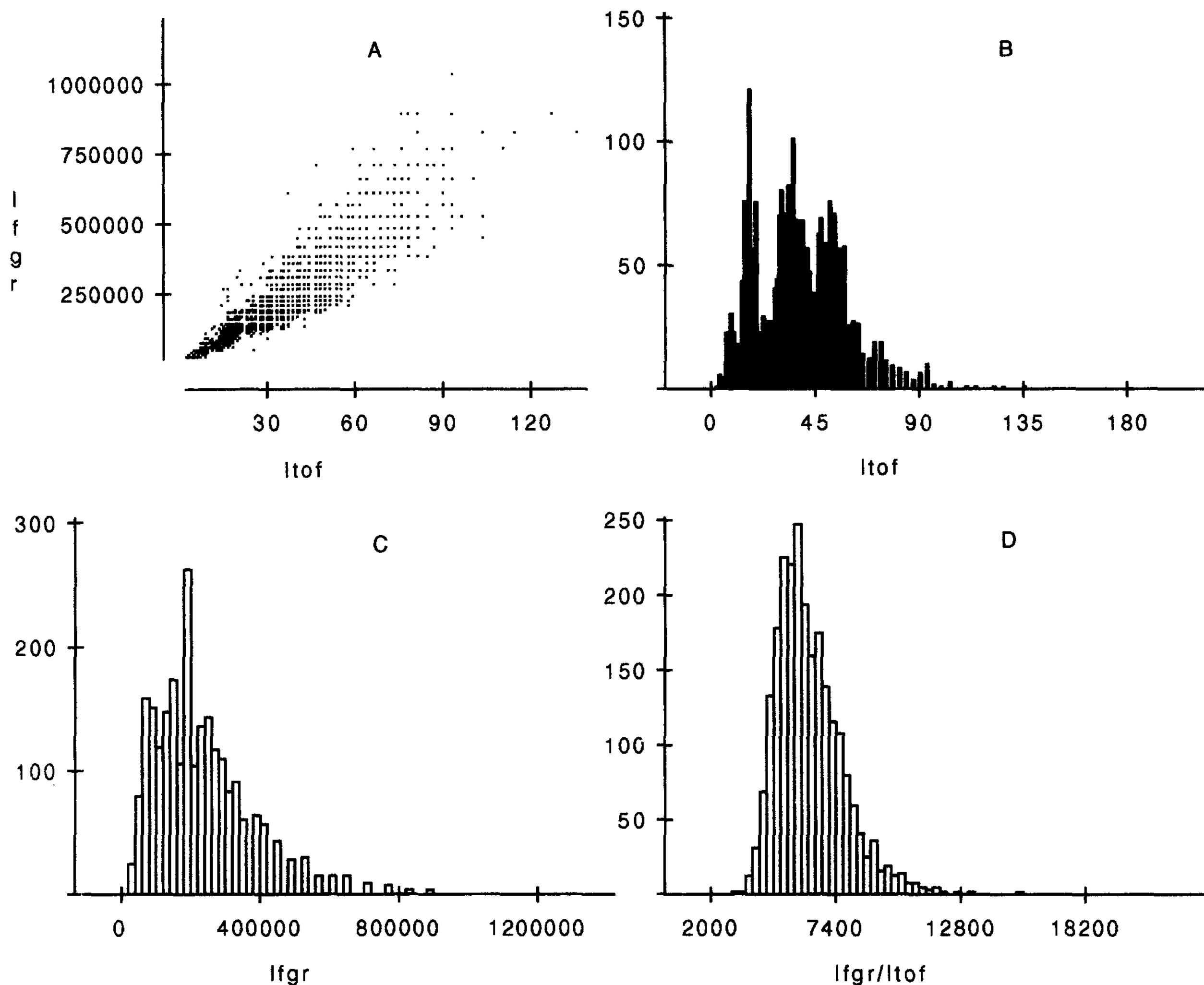

Fig. 5. Size distribution effect on flow cytometric measurement of low light-adapted *Skeletonema costatum*. (A) Bivariate plot of FGR and TOF; both parameters have been linearized. (B,C) Histograms of the linearized TOF and FGR, respectively. (D) Histogram of the ratio of the linearized FGR and TOF. Note the significant decrease in spread of the data and the more normal distribution of the data.

ences in average values that are obtained in the duplicate experiments are well below 5%.

The precision of the flow cytometric measurement can be estimated from measurements of fluorescent standard beads. For all parameters measured in this study the relative standard deviation obtained for chlorophyll standard beads is below 10%. This is significantly higher than the specified standard deviation (which is of the order of 3–5%). The layout of the optical plankton analyzer, which is designed for simultaneous measurement of particles in a wide size range, leads to a loss in precision. However, the standard deviations measured for the algae in one experiment are significantly higher than 10% due to biological variability (see Table III). In particular, for colony-forming phytoplankton species, such as *Anabaena* and *Skeletonema*, the standard deviation is very high; this is mainly attributable to differences in size of the individual algae, with a concomitant increase in pigment content and fluorescence intensity. The influence of size on the measurement is discussed in more detail below.

Table III also clearly shows the impressive dynamic range of the flow cytometer: *average* values ranging from 400 to 1,900,000 have been observed, spanning more than four decades. Linear values have been measured for individual particles that comprise an even larger dynamic range, spanning almost six decades. The very wide dynamic range implies that all particles of interest, with

Table IV. Ratios of Some Flow Cytometer Parameters (Values Are the Average of at Least 4000 Observations from Duplicate Experiments)

Species	Light field	FGR/FBR	FRR/FBR	FGO/FGR	FGR/PLS	FGO/PLS
Anabaena	High	0.39±0.15	1.0±0.3	0.10±0.02	0.58±0.28	0.07±0.07
	Low	1.1±0.5	1.7±0.7	0.20±0.06	1.4±0.4	0.28±0.10
Cryptomonas	High	0.46±0.10	0.16±0.04	1.0±0.1	0.81±0.20	0.81±0.26
	Low	0.67±0.13	0.22±0.05	1.2±0.2	2.2±0.7	2.6±1.0
Dunaliella	High	0.53±0.12	0.39±0.17	0.02±0.007	0.27±0.08	0.006±0.002
	Low	0.76±0.19	0.53±0.17	0.01±0.003	0.87±0.27	0.010±0.004
Isochrysis	High	0.54±0.16	0.18±0.04	0.03±0.008	1.1±0.4	0.03±0.01
	Low	0.84±0.15	0.24±0.03	0.01±0.004	3.3±1.3	0.05±0.02
Rhodomonas	High	1.0±0.2	0.12±0.03	12±2	2.3±0.8	27±9
	Low	1.1±0.2	0.18±0.05	9.3±0.9	4.3±1.2	39±11
Skeletonema	High	0.61±0.32	0.23±0.06	0.02±0.006	0.95±0.55	0.015±0.010
	Low	1.1±0.2	0.26±0.05	0.01±0.002	1.56±0.9	0.014±0.010
Synechococcus	High	5.9±3.4	1.0±0.06	3.6±1.0	6.1±5.4	21±17
	Low	3.8±1.9	0.88±0.21	9.3±2.8	3.3±2.1	29±18

sizes varying from 1–2 up to 500 μm, can be measured in one run.

It appears that the qualitative spectral information obtained in the fluorimetric measurements is confirmed by the flow cytometric data. In particular, the species that contain orange fluorescent pigments can be readily distinguished on the basis of their high FGO intensities. However, the more subtle effects of different excitation wavelengths are less easily recognized. The reason is that the optical parameters not only are determined by the pigment composition of the algae, but also are strongly influenced by their biological variability, in particular, by their physiology and size distribution.

The size of the algae leads to two effects. First, it causes differences in pigment content per particle and, hence, differences in the intensity of fluorescence and scatter light, in particular for colony-forming algae. Second, it leads to some additional signal as a result of the digital integration technique that is applied to increase the dynamic range of the flow cytometer. It is based on fast measurement and storage (every 200 ns) of the PMT signals. When the particle has passed, the accumulated signal is integrated and stored as a single value. The integration approach leads to increased signal intensities, also for parameters where only stray light is measured (e.g., FGO for non-phycobiliprotein-containing phytoplankton; see, e.g., the cluster of the bacillariophyte *Rhizosolenia* in Fig. 6). In the latter case the increase is due to prolonged integration of stray light and PMT dark counts. In the bivariate plots this effect can be observed as a sloping increase of the parameter with increasing TOF value.

Of course the differences in pigment content as a result of the phytoplankton size distribution have a stronger influence than the stray light effects. An increase in size is generally accompanied by an increase in light scatter, in particular, when the signal is integrated in time. Furthermore, the pigment content of the algae increases with size, which is readily recognized in the bivariate plots. Differences in pigment content affect mostly the fluorescence intensities that are measured, especially in colony-forming algae, such as *Anabaena* and *Skeletonema*. In noncolonial algae (in this study all except *Anabaena* and *Skeletonema*), the variability in the parameter values is determined by the pigment content and composition of the individual algae, as well as by the status of their photosynthetic apparatus (e.g., via photoinduced processes). Of course these characteristics of the individual species are subject to biological variability: the standard deviations for the fluorescence parameters for unicellular algae amount to 30–50% (see Table III). In this case the spread is determined by the size distribution to a small extent: in bivariate plots of, for instance, a fluorescent parameter vs TOF, invariably a slanting cluster is observed, with higher fluorescence values for the longer particles. Other effects, for instance, differences in pigment content as a result of light history, are much more important.

For colony-forming phytoplankton, such as *Skeletonema* and *Anabaena*, the size effect, however, is considerable. Both these algae form filamentous colonies, which are composed of a large number of single cells joined together in long strings. Figure 5 shows a bivar-

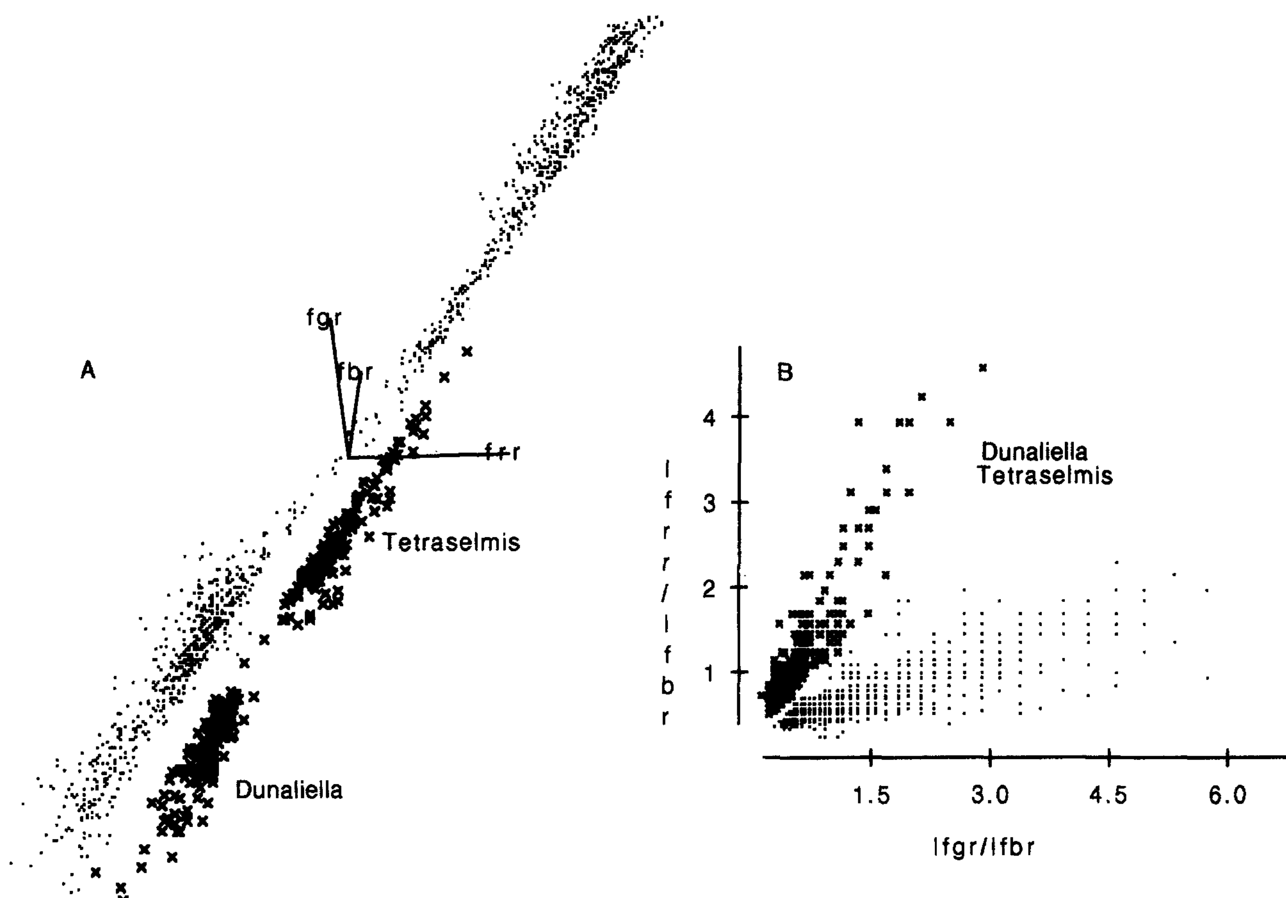

Fig. 6. Application of fluorescence ratios to discern chlorophytes and prasinophytes from a complex mixture: *Dunaliella* and *Tetraselmis* are clearly separated from the other species, due to their efficient absorption in the red.

iate plot obtained for *Skeletonema costatum* in which FGR is plotted against TOF. Both parameters have been linearized. The size distribution is clear, particularly from the TOF histogram shown in Fig. 5B. The first, small peak in the distribution is due to single cells (chloroplast size, about 9 μm). The next peaks clearly discernible are due to colonies with 2, 4, 6, 8, and 10 cells, respectively. The large size distribution leads to a significant spread in the fluorescence parameters as well (standard deviations of over 100% are observed; see Table III; note that, in particular, for colony-forming algae with an irregular size distribution such as *Skeletonema costatum*, the standard deviation is not well defined). To some extent the size effect can be removed by dividing the (linearized) intensity values by the TOF (see Fig. 5D). The standard deviation in the linear FGR is almost 70% (220,000 ± 140,000); after division—for each individual particle—by the linear TOF, the standard deviation decreases to less than 25% (6300 ± 1500). For *Anabaena* an even larger decrease is observed, as the size distribution is even more extended than for *Skeletonema*. One should consider, however, that the TOF is representative only of the length of the particles and does not provide a good measure of the size. This approach, therefore, can be applied only to filamentous colony-forming phytoplankton. It is interesting to note that, from the plot of the fluorescent parameter vs TOF, even the shape of the colony can be derived. In linear colonies, such as those formed by *Skeletonema* and *Anabaena*, the fluorescence signal is found to vary linearly with the length of the particle. Phytoplankton species such as *Phaeocystis pouchetii*, which form spherical colonies, show a third power dependence with the particle length for the intensity of the fluorescence signals (results not shown here).

The TOF values given in Table III are obtained by transformation of the logarithmic values that have been stored in list mode. The time that the FGR signal is above threshold is registered and taken as the TOF. Of course the TOF is determined by the convolution of the

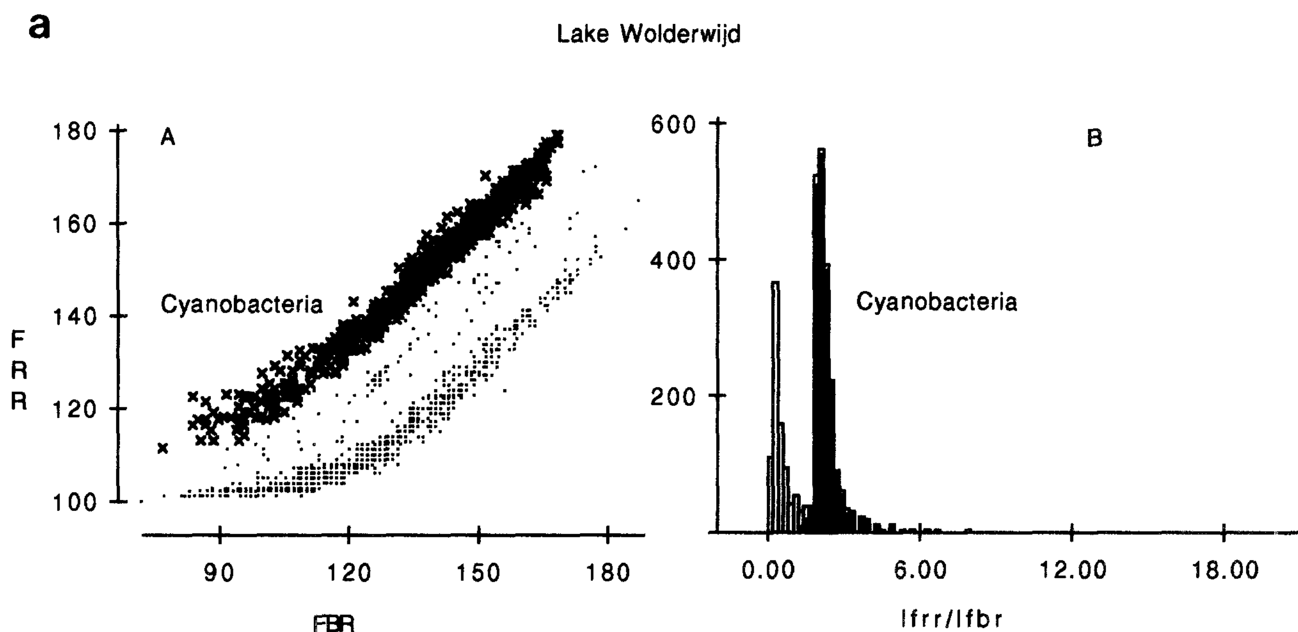

Fig. 7. Examples of flow cytometric analysis in the field. (a) Sample from the freshwater Lake Wolderwijd. The Cyanobacteria are straightforwardly identified from the bivariate plot by their high FRR. They are indicated in the plot by X. (b) Sample from the North Sea, 10 km off the coast of Noordwijk. Several clusters of orange fluorescing algae are visible: *Rhodomonas* (●) and *Mesodinium rubrum* (X) at even higher fluorescence intensities. The broad range at the bottom of the figure is due to bacillariophytes and dinophytes; they cannot be discerned on the basis of flow cytometry, unless specific staining is used. The *Rhizosolenia delicatula* cells are easily recognized due to their relatively large size.

red fluorescent part of the cells (the chloroplasts may be unevenly distributed over the cell) and the area that is irradiated by the Ar-ion laser. The laser focus is 5 μm wide at the waist (i.e., at the points where the laser intensity is $1/e^2$ of the maximum intensity). The transformation of the logarithmic TOF values consists of linearization and correction for the spatial distribution of the laser spot. The resulting TOF values are reliable only for larger cells. For instance, for *Synechococcus* an average value of about 0 is calculated for the TOF, which indicates that the size of these particles is well below 5 μm. In addition, the pigment content will influence the TOF: all dark-adapted algae with higher FGR show an increased average TOF. As the PLS values remain unchanged (for the unicellular algae), the difference in size distribution must be small. The difference therefore must be due to the triggering effects: the higher pigment content in the shade-adapted cells causes earlier and longer triggering of the measurement, as the threshold remains fixed. Therefore the TOF parameter can only be used as an estimate. At this moment other approaches to triggering are investigated, which may give a better representation of the particle length.

In addition, the absolute intensities of the fluorescence parameters are strongly influenced by photoadaptive effects. As indicated above, photadaptation leads to an increased pigment content. This is also indicated by the fact that the intensity of the fluorescence parameters is invariably higher for the cultures that have been grown under low light conditions. Interestingly, the 90° scatter (PLS) intensity is hardly affected by the light history of the algae, which indicates that the size of the phytoplankton is not changed as a result of photoadaptation effects.

Inspection of Table III indicates that it is not straightforward to discriminate phytoplankton classes just on the basis of absolute intensity measurements. Only the orange fluorescencing algae can be unambiguously identified. The other species have strongly varying intensity values that are attributable mainly to species-specific characteristics, such as size, pigment content (photoadaptation!), etc. The spectral properties that can be used to identify classes of phytoplankton species, as discussed above, cannot be clearly recognized. In particular, it is difficult to use differences in excitation efficiency as a function of wavelength. However, on the basis of the measurement of absolute values, also in field samples clusters can be identified in bivariate or trivar-

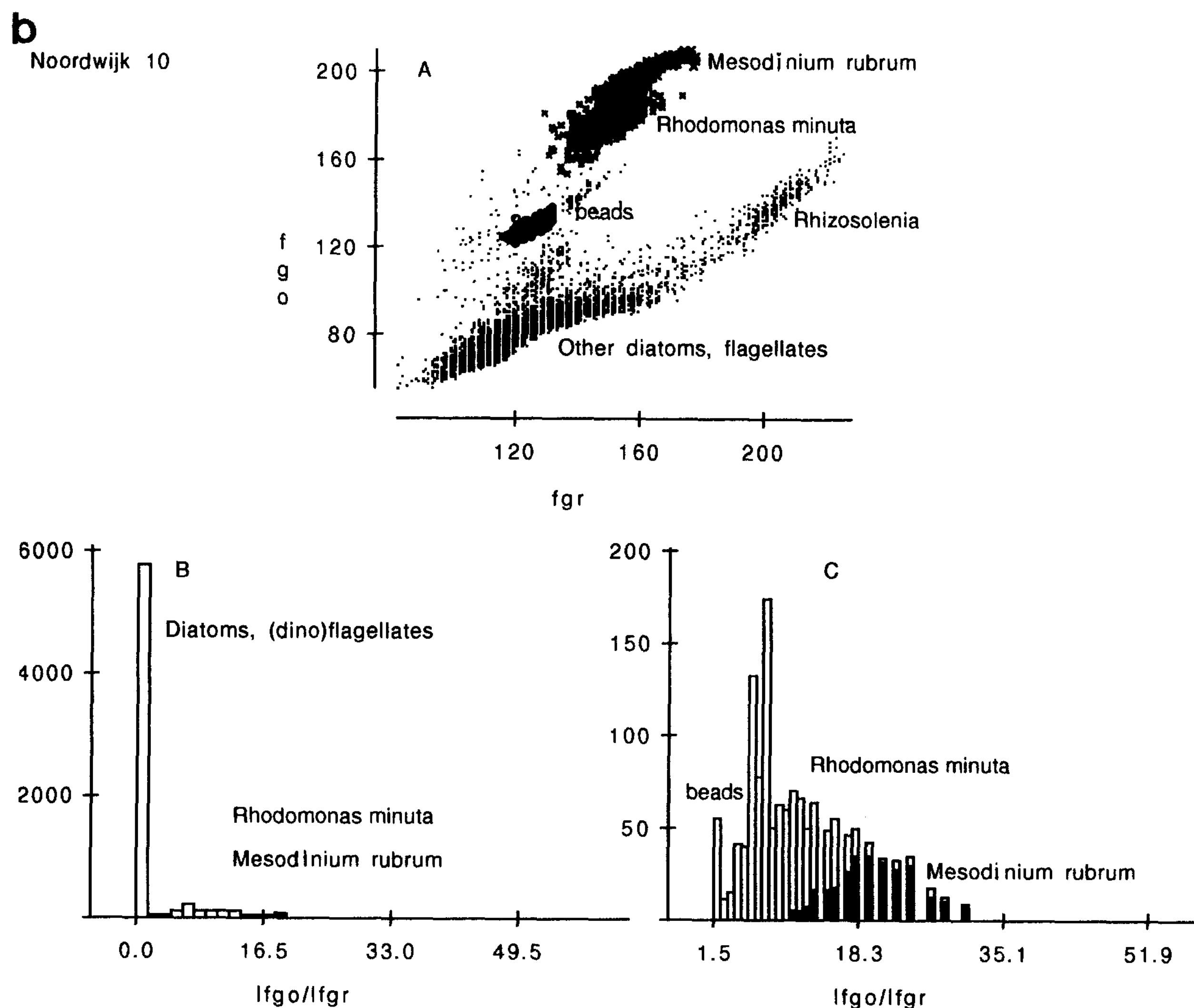

Fig. 7. Continued.

iate plots, which can be attributed to species that are present in the sample. Fortunately, in most field samples only one to five dominant species are present at the same time. Such identification can be done only by additional microscopic examination of selected cells (e.g., via sorting of cells that belong to the cluster, according to their optical properties).

Fluorescence Ratios

The application of absolute intensity measurements, described above, has several disadvantages. First, the intensities observed are determined to a great extent by the size effect. Second, the information from the pigment-specific excitation properties (i.e., the fluorescence intensities induced by the different laser wavelengths) cannot be easily visualized unless the effect is very large. Third, the number of parameters than can be examined at the same time is limited to two or three at the most. A promising approach to remove these disadvantages is the application of parameter ratios.

For bulk measurements using convential fluorometers ratioing of fluorescence intensities has already been applied. Yentsch and co-workers defined the chlorophyll-to-accessory pigments (CAP) ratio as the intensity of the chlorophyll fluorescence excited in the green (at 530 nm; i.e., in the carotenoid excitation region) divided by the intensity excited in the blue (at 450 nm; i.e., in the chlorophyll excitation region) [9–11]. The CAP ratio was found to have specific values for various

taxonomic phytoplankton groups. The ratioing approach in bulk spectrometry, however, has only a very limited potential, e.g., for the measurement of two species with very different and constant spectral differences, such as an orange and a red fluorescent alga, which are present at similar concentrations. In flow cytometry, however, a similar approach can be followed with much more success, because here fluorescence excitation ratios are obtained for individual algae. Apart from identification of the species, the flow cytometric measurements can also be used to study the interspecies variability.

Table IV shows a number of fluorescence intensity ratios that have been obtained for the species used in this study for cultures grown under both light regimes. Of course the logarithmic values were linearized before the ratio was calculated.

On the basis of the FGO/FGR ratio the orange fluorescent algae are readily identified. The size effect obviously is not present in the ratioed values. The species that contain only chlorophyll-a as fluorescent pigment show values of FGR/FGO that are all significantly lower than 0.1. The phycocyanin-containing *Anabaena* shows a somewhat higher ratio (0.1–0.2); this pigment has its fluorescence onset at about 600 nm, so that a small part of the emission will be measured in the orange channel. *Cryptomonas*, with the more blue-shifted phycoerythrin PE 565, shows ratios varying from 1 to 1.2. The two species with phycoerythrin PE 545, which has its emission maximum in the center of the wavelength region measured in the FGO channel, *Rhodomonas* and *Synechococcus*, have the highest FGO/FGR ratios: they far exceed 1.

The distinction that can be made on the basis of the FGR/FBR ratio, which is more or less comparable to the CAP ratio introduced by Yentsch *et al.*, is disappointing [9–11]. Many species, in particular, the bacillariophytes, dinophytes, and haptophytes, which are most common in the North Sea, contain accessory pigments that absorb in the green. In addition; the FGR/FBR ratio appears to depend strongly on the light history of the algae. Consequently, even the chlorophyte Dunaliella, which lacks strong green absorbing pigments, cannot be positively identified on the basis of the FGR/FBR ratio.

The FRR/FBR ratio is more conclusive for the determination of the chlorophyll-b-containing *Dunaliella*. Chlorophyll-b strongly absorbs in the 640-nm region that is excited by the HeNe laser. Also, the phycocyanin-containing *Anabaena* is straightforwardly identified on the basis of its high FRR/FBR ratio. This is a very important observation, as many nuisance freshwater algae belong to the same class as *Anabaena*.

All shade-adapted phytoplankton species show increased excitation efficiencies in the accessory pigment region, as compared to the cultures that were grown under high light intensity. This is obvious in Table IV from the FGR/FBR ratios, which are invariably higher for the low light-adapted cultures. The orange fluorescence (FGO) also is found to be significantly increased in the shade-adapted cultures, in the phycobiliprotein-containing phytoplankton (cf. FGO/FGR or FGO/FBR; not shown). The FRR/FBR ratio is particularly increased for the phycocyanin-containing *Anabaena*.

In addition, also the absolute fluorescence intensity levels are found to be higher for the cultures grown under low light conditions. This was indicated by the values given in Table III, but also the FGR/PLS and FGO/PLS ratios in Table IV indicate the increase in pigment content in shade-adapted phytoplankton. The PLS intensity is not strongly affected by photoadaptive effects, as discussed before. Therefore this parameter is eminently suited to correct for small instrument fluctuations and size effects.

The main advantage of the ratioing technique is that the size dependence of the parameters is removed, so that more objective and statistically sound classification can be done. Especially, the colony-forming algae illustrate this point: the absolute data show a tremendous variability, due mainly to the size effect, which has been eliminated as a result of the ratioing. In addition, the wavelength dependence of the excitation efficiency can be taken into account, which gives an additional handle for the classification of phytoplankton species.

The data presented in Table IV are obtained for cultures grown under extreme light conditions. Even when these conditions are taken into account, parameter ratios obtained give conclusive information on the phytoplankton pigment composition of the individual algae that have been measured. For the phytoplankton species that contain only chlorophyll-a as fluorescent pigment, classification is based on wavelength-dependent differences in excitation efficiency. In this way chlorophyll-b-containing green algae (chlorophytes, prasinophytes) can be discerned on the basis of enhanced excitation in the red part of the spectrum. The phytoplankton classes with enhanced excitation in the green region can be discerned from the other classes. Unfortunately no direct discrimination can be made between the two main representatives of the classes with green absorbing accessory pigments, the bacillariophytes and dinophytes, which are very abundant in coastal seawater. Discrimination of phycobiliprotein-containing phytoplankton is the easiest. On the basis of the FGO/FGR ratio, even the actual pigment composition of these algae can be derived (i.e., a ratio >2 for phycoerythrin PE 545-containing algae,

a ratio of about 1 for PE 565-containing algae, and a slightly but significantly increased ratio of 0.1–0.2 for phycocyanin-containing algae). The latter type of algae, to which many cyanobacteria belong that cause problems in freshwater lakes and hence draw considerable attention, is readily identified when the FRR is taken into account. The (allo)phycocyanins have strong absorption in the red. The considerations discussed above are commented upon in more detail in the next section, in which some applications of flow cytometric fluorescence measurements for identification of algae are discussed.

Applications

A few examples of identification of phytoplankton classes in complex samples are discussed to illustrate the possibilities of simple enumeration of algae based upon their fluorescence properties. Before presenting the examples it is good to emphasize that the aim of the application of flow cytometry in the analysis of phytoplankton is not to provide a detailed picture of all species present in the sample. This information can be gathered only at the expense of a tremendous effort in time by detailed microscope analysis. The flow cytometer should afford the fast enumeration of the (in general, only one to five) dominant species in the sample. In addition, for monitoring purposes, it is convenient to have a fast and reliable technique for enumeration of relative amounts of main phytoplankton classes, such as cyanobacteria in fresh waters or bacillariophytes, dinophytes, and cryptophytes in seawater.

In Fig. 6 a three-dimensional (3D) plot is shown of the flow cytometric data obtained for a complex mixture consisting of 10 algal species. In the 3D plot two species can be discerned that show elevated FRR emission. The prasinophyte *Tetraselmis* and the chlorophyte *Dunaliella* can be discerned on the basis of their efficient absorption in the red, due to their high chlorophyll-b content. Much easier quantification of these two algal species can be done by making use of fluorescence ratios, as can be inferred from Fig. 6B. The two chlorophyll-b-containing algae can be clearly discerned from the other algal species, which show a significantly lower FRR/FBR ratio. The other algae in the sample are mostly bacillariophytes, which all give similar fluorescence properties. The only way to discriminate the bacillariophytes is via their size, e.g., by making use of the TOF measurement or the intensity of fluorescence intensity differences, or via their shape, by making use of scatter measurements.

Two examples of flow cytometric data that have been obtained for phytoplankton in field samples are shown in Fig. 7. Figure 7a shows a bivariate plot of a freshwater sample. In the summer, eutrophic lakes in the Netherlands, such as Lake Wolderwijd where this sample was taken, show extremely high concentrations of cyanobacteria. The main bloom-forming species is *Microcystis aeruginosa*. This alga contains gas vacuoles and, in particular circumstances, may start to float and to form a green layer covering the water surface. In addition, blooms of the toxin-containing cyanobacterium *Anabaena flos-aquae* may occur. Hence, the Water Authorities put a lot of effort into the monitoring of the abundance of cyanobacteria. Flow cytometry appears very well suited for this application: the relevant cyanobacteria all contain the pigment phycocyanin, which can be readily discriminated on the basis of its very efficient excitation in the red part of the spectrum. Figure 7a shows clear distinction of the cyanobacteria (indicated by X) from the other species by their significantly higher FRR intensities. The cyanobacteria in this sample are predominantly filamentous colony-forming *Anabaena flos-aquae*, as can be inferred from the large spread of the absolute fluorescence intensities. When the FRR/FBR parameter ratio is calculated for the individual particles, a histogram is obtained from which the cyanobacteria can be very easily quantified, due to their increased FRR intensity.

The bivariate plot shown in Fig. 7b was obtained for a sample taken from a standard monitoring point in the North Sea, 10 km off the Dutch coast. In the Dutch coastal zone, bacillariophytes (diatoms) are predominant during the main part of the year. The diatoms do not contain any orange fluorescent pigments and are, therefore, located in the bottom part of the figure. The larger diatoms, with higher values of FGR, appear as a slanting feature due to the integration of stray light. The figure also shows several clusters that do have high values for FGO, which indicates the presence of phycobiliproteins. The species can be straightforwardly identified as *Rhodomonas minuta*, a cryptophyte, and *Mesodinium rubrum* by microscopic analysis. The latter species is heterotrophic but occurs in symbiosis with very small orange fluorescent phytoplankton. Orange fluorescent phytoplankton, in particular *Rhodomonas*, is a major constituent of the Dutch coastal waters throughout the year and can be easily identified by means of flow cytometry. Again, analysis is facilitated by making use of a fluorescence ratio, in this case FGO/FGR. The phycobiliprotein-containing algae show clearly increased ratios, as compared to the much more numerous bacillariophytes and dinophytes. Unfortunately, the latter two phytoplankton classes cannot be separated by means of fluorescence measurements, as they have accessory pigments with similar spectral properties.

In the above attention has been paid to the occurrence of photoadaptive effects in the phytoplankton fluorescence characteristics. The consequence of the photoadaptation effect could be that flow cytometric data for field samples cannot be easily interpreted in terms of simple relations between parameters, or specific regions in the parameter space, which would be characteristic for particular phytoplankton species. In practice, photoadaptive effects do not play an important role, as can be derived from measurements of *Rhodomonas* species throughout the year [22]. The approach that is followed to remove light-induced effects as much as possible from the flow cytometric data is to measure samples that have been taken at 3 m below the water surface and have been stored for several hours in the dark at reduced temperatures. Further study of samples taken at several depths, with different light histories, may be worthwhile. Of course, the flow cytometric data also can be used to determine photoadaptive effects for specific species.

CONCLUSIONS

Spectral characteristics of phytoplankton species can be determined flow cytometrically by means of combined multilaser excitation and multiwavelength detection. On the basis of such measurements several important groups of phytoplankton can be distinguished ataxonomically, on the basis of differences in pigment composition that are apparent in their spectral characteristics. Examples are the selective determination of cyanobacteria, of cryptophytes, and of chlorophytes and prasinophytes. Although the parameter values change in a relative as well as in an absolute manner as a result of photoadaptation, this rough distinction can also be made for field samples, provided that care is taken to remove spurious light-dependent effects. The observed data can even be used for interpretation of the light history of the phytoplankton. The main phytoplankton constituents of the Dutch coastal waters, however, cannot be easily distinguished on the basis of spectral characteristics: the bacillariophytes and the dinophytes contain spectrally similar accessory pigments.

Further improvements of the classification may be achieved by application of alternative interpretation techniques. An example is the neural network approach, which allows one to make use of all eight parameters simultaneously to achieve more subtle discrimination of selected species [23]. Also, selective chemical staining of phytoplankton species with fluorescent labels can be applied; an example is the staining of dinophytes with proflavine [24]. Finally, *species-specific* labeling of phytoplankton via immunochemical techniques can be applied [25].

REFERENCES

1. T. E. Whitledge and C. D. Wirick (1983) *Deep-Sea Res.* **30**, 297–309.
2. T. E. Whitledge and C. D. Wirick (1986) in J. Bowman, C. M. Yentsch, and W. T. Peterson (Eds.), *Lecture Notes on Coastal and Estaurine Studies, Vol. 17. Tidal Mixing and Plankton Dynamics*, Springer Verlag, Berlin, pp. 449–462.
3. J. W. Hofstraat (1990) *Netherlands J. Photon.* **19**(4), 4–18.
4. J. W. Hofstraat, W. J. M. van Zeijl, J. C. H. Peeters, L. Peperzak, and G. B. J. Dubelaar (1990), in H. O. Nielsen (Ed.), *SPIE Proceedings, Vol. 1269, Environment and Pollution Measurement Sensors and Systems*, SPIE, The International Society for Optical Engineering, Bellingham, WA, pp. 116–133.
5. L. Legendre and C. M. Yentsch (1989) *Cytometry* **10**, 501–511.
6. D. A. Phinney and T. L. Cucci (1989) *Cytometry* **10**, 511–522.
7. J. C. H. Peeters, G. B. J. Dubelaar, J. Ringelberg, and J. W. M. Visser (1989) *Cytometry* **10**, 522–529.
8. G. B. J. Dubelaar, A. C. Groenewegen, W. Stokdijk, G. J. van den Engh, and J. W. M. Visser (1989) *Cytometry* **10**, 529–540.
9. C. S. Yentsch and C. M. Yentsch (1979) *J. Mar. Res.* **37**, 471–483.
10. C. S. Yentsch and D. A. Phinney (1985) in A. Zirino (Ed.), *Mapping Strategies in Chemical Oceanography, Advances in Chemistry Series No. 209*, American Chemical Society, Washington, DC, pp. 259–274.
11. C. S. Yentsch and D. A. Phinney (1985) *J. Plankton Res.* **7**, 617–632.
12. J. Beeler SooHoo, D.A. Kiefer, D. J. Collins, and I. S. McDermid (1986) *J. Plankton Res.* **8**, 197–214.
13. J. Hilton, E. Rigg, and G. Jaworksi (1989) *Freshwater Biol.* **21**, 375–382.
14. J. Hilton, E. Rigg, and G. Jaworski (1989) *J. Plankton Res.* **11**, 65–74.
15. R. J. Olson, E.R. Zettler, and O.K. Anderson (1989) *Cytometry* **10**, 636–644.
16. S. P. Chu (1942) *J. Ecol.* **30**, 284–325.
17. L. van Liere and L. R. Mur (1978) *Mitt. Internat. Ver. Limnol.* **21**, 158–167.
18. R. R. L. Guillard (1975) in W. L. Smith and M. H. Chanley (Eds.), *Culture of Marine Invertebrate Animals*, Plenum, New York.
19. M. E. J. de Vreeze and J. W. Hofstraat (1991), unpublished results.
20. J. W. Hofstraat, K. Rubelowsky, and S. Slutter (1992) *J. Plankton Res.* (in press).
21. A. Neori, O. Holm-Hansen, B. G. Mitchell, and D. A. Kiefer (1984) *Plant Physiol.* **76**, 518–524.
22. J. W. Hofstraat, W. J. M. van Zeijl, and M. Rademaker (1992), to be published.
23. H. W. Balfoort, J. Snoek, J. R. M. Smits, L.W. Breedveld, J. W. Hofstraat, and J. Ringelberg (1992) *J. Plankton Res.* (in press).
24. J. W. Hofstraat, J. Newell, W. J. M. van Zeijl, and M. L. Latuhihin (1991) unpublished results.
25. E. G. Vrieling, W. W. C. Gieskes, F. Colijn, J. W. Hofstraat, L. Peperzak, and M. Veenhuis (in press).