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Routine quantification of phytoplankton groups—microscopy or pigment analyses?

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ABSTRACT: Phytoplankton pigments in samples taken from nutrient-enriched and non-enriched 3 m^3 seawater enclosures were separated and quantified using high-performance liquid chromatography (HPLC). The enclosures were with and without inorganic (N, P, Si) and organic (glucose, C) nutrient enrichments, resulting in a variation of phytoplankton groups in time and space. The relative contribution of the major phytoplankton groups to the total chlorophyll *a* (i.e. chlorophyll *a* plus chlorophyllide *a*) was estimated by the CHEMTAX program. The results were compared to phytoplankton groups identified and quantified by light and epifluorescence microscopy. For the pigmented flagellate groups the results obtained by microscopy and pigment analyses using the CHEMTAX program showed similar trends. The picocyanobacteria were readily quantified by microscopy and the results were similar to those obtained by flow-cytometry, while the CHEMTAX program for the cyanobacteria revealed different trends. Microscopy and pigment analyses provided similar trends in diatom population development. Estimated diatom contributions to total phytoplankton biomass, however, were considerably higher when based on microscopy than when based on the CHEMTAX program, especially in Si-amended enclosures. Total chlorophyll *a*:carbon ratios for diatoms were at the lower end of a previously reported range between 1:27 and 1:67. For the pigmented flagellate groups the total chlorophyll *a*:carbon ratios were above that range. In routine monitoring of phytoplankton we recommend the use of the CHEMTAX program based on HPLC pigment analyses accompanied by a screening for the dominating species by microscopy, and by flow-cytometry for quantification of picocyanobacteria.

KEY WORDS: HPLC pigment analyses · CHEMTAX program · Microscopy · Chlorophyllide *a* · Chlorophyll *a*:carbon ratios · Pigmented flagellates · Cyanobacteria · Diatoms

INTRODUCTION

During the last few decades, phytoplankton has been monitored in an increasing number of marine environments. Emphasis has been put on the occurrence of potentially harmful species and on seasonal succession of algal groups, their diversity, magnitude, and duration to monitor environmental changes (Pybus 1996, Gobler & Sañudo-Wilhelmy 2001, Edwards et al. 2002). Phytoplankton monitoring has been used as an early warning system for potentially harmful algal blooms (Dahl & Johannessen 1998) and has resulted in indications of climatic changes, e.g. glacial melting in the Antarctic, where an increased dominance of cryptophytes was used as a sign of an increased input of melting water into the coastal regions of the Southern Ocean (Moline & Prézelin 1996).

Phytoplankton enumeration using the Utermöhl method has been in use since the early 1930s (Utermöhl 1931). In the 1970s, a simplified method, whereby only 90% of the phytoplankton biomass is assessed, came into use (Willén 1976), and the time for analysis

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decreased. Routine analyses are usually performed using preserved liquid samples in which the chlorophyll *a* (chl *a*) is degraded and species identification is only possible by morphological characteristics, making it often impossible to discriminate between photoautotrophic and heterotrophic flagellates. In contrast, samples taken for epifluorescence microscopy are filtered and frozen on the sampling day, which preserves the chl *a* fluorescence. Using epifluorescence microscopy, phytoplankton cell shapes, flagella, or other cell extensions can be observed even in unstained preparations, together with the size and shape of the chloroplasts and their red or orange autofluorescence, which provide the basis for identification of pigmented flagellate groups (Havskum & Riemann 1996).

Given the patchy distribution and inter-annual variability of phytoplankton (Smayda 1998), statistically significant changes can only be revealed when a large number of samples is analysed. Traditional methods, however, based on the Utermöhl method and/or analyses using epifluorescence microscopy, are very time-consuming. In addition, analyses of phytoplankton communities by microscopy depend on the taxonomic skills of the operator and are associated with counting errors and errors in measuring cell size, resulting in a large variability in sample analyses between laboratories (Duarte et al. 1990).

In contrast, analyses of phytoplankton pigments by HPLC are more reproducible than microscopic analyses (Schlüter et al. 2000), and are increasingly being used for determining the composition of phytoplankton communities (Tester et al. 1995, Wright et al. 1996, Loret et al. 2000). The CHEMTAX program was developed by Mackey et al. (1996) as a data treatment programme for calculating algal class biomass as chl *a* from the concentrations of the phytoplankton accessory pigments determined by HPLC. The CHEMTAX program uses factor analysis to find the best fit to the data, based on suggested pigment:chl *a* ratios for the phytoplankton groups to be determined as chl *a* (Mackey et al. 1996).

In the present investigation, thorough phytoplankton group quantifications were performed on water samples collected from nutrient-manipulated 3 m^3 seawater enclosures. The objective was to evaluate pigment analyses and subsequent data treatment by the CHEMTAX program (Mackey et al. 1996) by comparing the results with those obtained by microscopy. Because phytoplankton group-quantification of fixed, liquid samples using the Utermöhl method (Utermöhl 1931, 1958) makes it difficult or impossible to identify many pigmented flagellate groups, epifluorescence microscopy was also employed in the present investigation. In addition, total chl *a* was measured in a traditional way, i.e. using fluorometry, and picocyanobacteria were enumerated using flow-cytometry.

MATERIALS AND METHODS

Study site. For this study, 5 polyethylene enclosures were filled on 15 June 1998 with 3 m^3 unfiltered surface water from Isefjord, Zealand, Denmark. The enclosures were arranged along a north–south axis, and water-column homogeneity was ensured by an air-lift system (Egge & Heimdal 1994). Water samples were taken daily during 11 d between 08:00 and 09:00 h using 2 m long tubes. The initial silicate concentration was 5 mmol m^{-3}, while PO_4-P and NO_2+NO_3-N were below 0.16 and 0.36 mmol m^{-3}, respectively (Havskum et al. 2003). In order to achieve differences in phytoplankton development, different manipulations with nitrate (N), phosphate (P), glucose (C), and silicate (Si) were performed: +NP, +NPC, +NPSi, +NPCSi and no addition. Nutrients were added daily between 21:00 h and 22:00 h during the period 16 to 25 June 1998. N was added as $NaNO_3$ (1.6 mmol m^{-3}), and P as KH_2PO_4 (0.1 mmol m^{-3}); 2 enclosures (+NPC and +NPCSi) received an additional 106 mmol m^{-3} C (added as glucose) to favour bacterial growth, and 2 enclosures (+NPSi and +NPCSi) received between 3.2 and 9.2 mmol m^{-3} Si (added as Na_2SiO_3 $9H_2O$) to favour diatom growth when the ambient silicate concentrations in these enclosures fell below 3 mmol m^{-3}. Nutrient analyses were performed daily, immediately after taking the water samples. NO_2^-, NO_3^- (including NO_2^-), NH_4^+, PO_4^{3-} and SiO_4^- concentrations were determined using an autoanalyser following Grasshoff et al. (1983). For further details concerning the experimental set-up and microbial dynamics see Jacquet et al. (2002) and Havskum et al. (2003).

Determination of total chl *a* by fluorometry. Total chl *a* was measured fluorometrically using, essentially, the procedure of Parsons et al. (1984). Samples (25 or 5 ml) were collected on 25 mm GF/F glass-fibre filters and extracted overnight in 90% acetone, in darkness and at 4°C (Parsons et al. 1984). Filtration time never exceeded 5 min under low pressure (<5 mm Hg). Readings were performed on a Turner Designs fluorometer calibrated spectrophotometrically with a mixture of water obtained from different treatments including the *in situ* natural community. For the calibration, samples (500 to 700 ml) were collected on 25 mm GF/F filters and extracted in 3 to 4 ml of 90% acetone as described above. The extract was analysed in a spectrophotometer and the total chl *a* concentrations (µg ml^{-1}) calculated following the equations given by Jeffrey & Humphrey (1975), i.e. total chl $a = 11.85E_{664} - 1.54E_{645} - 0.08E_{630}$, where E stands for the absorbance of the sample at 664, 645 and 630 nm, respectively (previously corrected for absorbance at 750 nm). Once the total chl *a* concentration was known, 4 different dilutions (ca. 1:10, 1:100, 1:1000 and

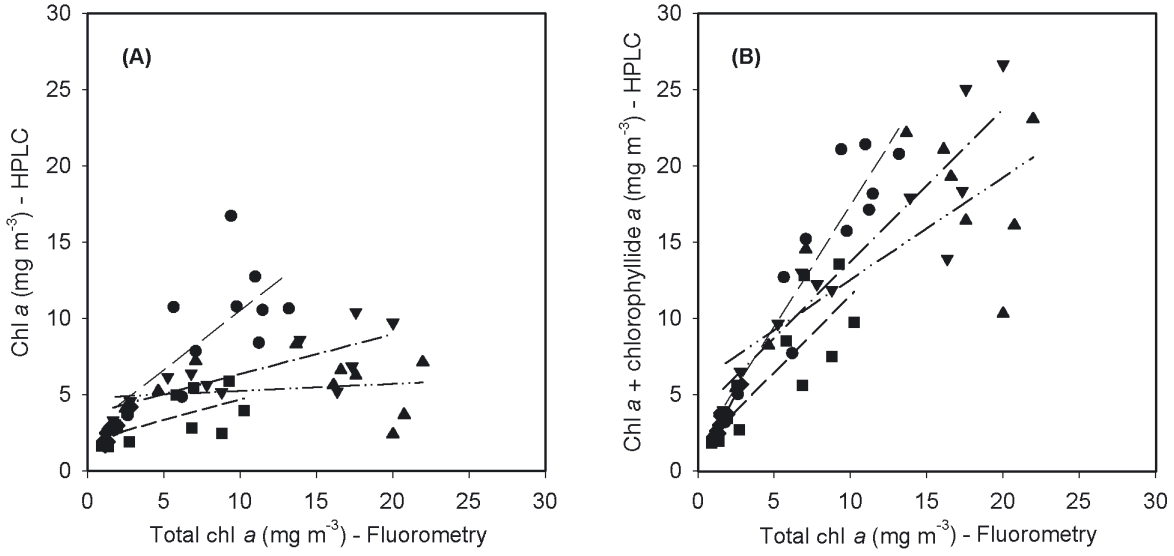
1:10 000) of each extract were made; these were read at 4 different sensitivities (scales) of the fluorometer. In this way, each reading allowed the calculation of a conversion factor (which takes into account the scale of the fluorometer) from fluorescence values to total chl *a* concentration as follows: conversion factor = (total chl *a* concentration of the extract) × (dilution of the extract) × (fluorometer scale) / (flurometer reading-blank); 90% acetone was used as blank, and read at each scale of the fluorometer. An average conversion factor (i.e. 0.10 ± 0.004) was obtained from the 12 fluorometric readings as described above.

Pigment analyses using HPLC. 100 to 500 ml samples were filtered (filtration time < 15 min) in dim light to prevent photodegradation (Moreth & Yentsch 1970) onto 25 mm GF/F filters; these were subsequently frozen, stored in liquid nitrogen and analysed within 3 mo. Prior to analysis, the filters were thawed, placed in 3 ml 90% acetone to prevent loss of hydrophilic pigments (Latasa et al. 2001), sonicated on ice and extracted at 4°C for 24 h. The samples were refiltered using disposable syringes and 0.2 µm Teflon syringe filters, and injected into a Shimadzu LC-10A HPLC system according to the method described by Wright et al. (1991). The HPLC system was calibrated with pigment standards from The International Agency for ^{14}C Determination, DHI Water & Environment, Denmark. Peak identities were routinely confirmed by online diode array.

Use of CHEMTAX program. The biomass of the phytoplankton groups was calculated by CHEMTAX (Mackey et al. 1996) by loading the concentration of the pigments detected by HPLC and the pigment ratios in Schlüter et al. (2000) for phytoplankton species common in estuaries and coastal areas. Since the samples in the present investigation were integrated by depth, the average ratios of the 3 light intensities given in Schlüter et al. (2000) were used. The pigments loaded were peridinin, 19′-butanoyloxyfucoxanthin (19′-but), 19′-hexanoyloxyfucoxanthin (19′-hex), fucoxanthin, neoxanthin, prasinoxanthin, violaxanthin, alloxanthin, zeaxanthin, chl *b*, and total chl *a* (chl *a* plus chlorophyllide *a*). By calculating the ratios of prasinoxanthin:chl *b*, lutein:chl *b*, and zeaxanthin:chl *b* on the data sets according to Schlüter & Møhlenberg (2003), the presence of prasinophytes without the diagnostic pigment prasinoxanthin and the sporadic presence of cyanobacteria could be proved, while the presence of chlorophytes could be excluded. Furthermore, size-fractionations of the phytoplankton revealed that 2 types of haptophytes were present: Type 3 with 19′-hex and Type 4 with both 19′-hex and 19′-but (Schlüter & Møhlenberg 2003). Based on these considerations and the diagnostic pigments detected, 8 algal groups were loaded in CHEMTAX: dinoflagellates, prasinophytes with prasinoxanthin, prasinophytes without prasinoxanthin, cryptophytes, Haptophytes Type 3, Haptophytes Type 4, diatoms, and cyanobacteria (see Table 1).

Total chl *a* was used to estimate total phytoplankton biomass, because it is the most commonly used indicator (Holm-Hansen et al. 1965, Jeffrey & Humphrey 1975, Colijn & Reckermann 2000). During the filtration process, chlorophyllases, which are present in many algal species, may become activated and degrade chl *a* to chlorophyllide *a*. This process is especially common in diatoms (Jeffrey & Hallegraeff 1987 and references herein). Because the maximum fluorescence spectra of chl *a* and chlorophyllide *a* are almost identical in 100% acetone (excitation at 430 and 426 nm, emission at 668 and 667 nm; Jeffrey et al. 1997), total chl *a* measured fluorometrically includes both chl *a* and chlorophyllide *a*. HPLC analysis, however, clearly distinguishes both compounds. Since chlorophyllide *a* is considered an experimental artefact, it is added to the HPLC chl *a* to account for this problem (Latasa & Bidigare 1998). We refer to 'chl *a* plus chlorophyllide *a*' as 'total chl *a*' to avoid confusion with standard nomenclature. We used total chl *a* measured by HPLC in the CHEMTAX program in order to calculate the contribution of each algal group. In the present investigation, total chl *a* measured by fluorometry was not significantly correlated to chl *a* measured by HPLC for data from the enclosures +NPC and +NPSi ($r_{+NPC} = 0.6018$, $p_{+NPC} = 0.0501$, $r_{+NPSi} = 0.179$, $p_{+NPSi} = 0.599$) (Fig. 1A). Data from the remaining 3 enclosures were significantly correlated ($p < 0.05$; $r_{+NP} = 0.704$, $r_{+NPCSi} = 0.771$, $r_{no\ addition} = 0.903$) (Fig. 1A). In contrast, all correlations between fluorometry measurements of total chl *a* and HPLC measurements of total chl *a* were significantly correlated ($p < 0.05$; $r_{+NP} = 0.919$, $r_{+NPC} = 0.832$, $r_{+NPSi} = 0.741$, $r_{+NPCSi} = 0.919$, $r_{no\ addition} = 0.941$) (Fig. 1B). Total chl *a* measured by fluorometry had always a closer 1:1 relationship to total chl *a* measured by HPLC than to chl *a* measured by HPLC alone.

Determination of pigmented flagellate and picocyanobacteria biomass by epifluorescence microscopy. Samples were fixed with glutaraldehyde (final concentration 2%), which gave the cells a greenish stain, and subsequently filtered onto black polycarbonate filters (pore size 0.2 µm for enumeration of picocyanobacteria, and 0.8 µm for enumeration of pigmented flagellates) (Porter & Feig 1980). The filtered volume varied between 1 and 50 ml. When diatoms were dominating, up to 10 subsamples were filtered in order to assure an adequate distribution of phytoplankton cells on each filter (i.e. cells did not lie on top of each other). The filters were frozen on the sampling day and stored at −30°C to preserve the chl autofluorescence (Porter & Feig 1980, Bloem et al. 1986, Sanders et al. 1989), and were viewed within 6 mo in



Enclosures		(A) b(1), b(0), r^2	(B) b(1), b(0), r^2
+NP	●	0.78, 2.73, 0.50	1.59, 1.48, 0.84
+NPC	■	0.27, 2.01, 0.36	1.01, 1.42, 0.69
+NPSi	▲	0.05, 4.80, 0.03	0.67, 5.89, 0.55
+NPCSi	▼	0.26, 3.75, 0.60	1.00, 3.74, 0.84
No addition	◆	1.25, 0.66, 0.82	1.92, 0.26, 0.89

Fig. 1. Total chlorophyll *a* (chl *a*) measured by fluorometry in relation to (A) chl *a* measured by HPLC, and (B) total chl *a* (i.e. chl *a* plus chlorophyllide *a*) measured by HPLC. Table below figure shows symbols for enclosures and linear regression coefficients. All data were significantly positively correlated, except data for enclosures +NPC and +NPSi in (A)

an epifluorescence microscope with 600 or 1000× magnification. The organisms were assigned to 11 groups according to their size and taxonomic relationships (Havskum & Hansen 1997). The biomass of groups belonging to the same algal class were eventually combined in order to compare the results obtained by microscopy with those obtained by pigment analyses using the CHEMTAX program. At least 100 cells of each group in each sample were counted. Cell volume was calculated from the average cell dimensions of 100 cells in each group. The dimensions of naked flagellates were multiplied by a factor of 1.1 to compensate for shrinkage due to fixation (Choi & Stoecker 1989). Cell volume (μm^3) was converted to cell carbon (pg) using conversion factors: 0.22 pg C μm^{-3} for picocyanobacteria (Søndergaard et al. 1991), 0.13 pg C μm^{-3} for thecate dinoflagellates, 0.11 pg C μm^{-3} for all other flagellates including athecate dinoflagellates (Mullin et al. 1966, Strathmann 1967).

Determination of diatom biomass. Samples were fixed with formaldehyde buffered with hexamine (0.6% final concentration) and diatom biomass was determined using the Utermöhl technique (Utermöhl 1958). Cell carbon was calculated from cell volume after subtracting the vacuole volume (Hillebrand et al. 1999), assuming a plasma layer thickness of 1 µm (Strathmann 1967), and the remaining volume (μm^3) was converted to carbon content (pg) using the factor 0.11 pg C μm^{-3} (Mullin et al. 1966). For identification and enumeration, an inverted light microscope was used. Diatoms were assigned either to 1 of 3 dominating species (*Skeletonema costatum* [Greville] Cleve, *Dactyliosolen fragilissimus* [Bergon] Hasle comb. nov. or *Chaetoceros curvisetus* Cleve), or to an 'other diatoms' group. As 1 of the dominant species (*S. costatum*) displayed varying cell dimensions, individual cells were assigned to 3 subgroups according to their cell size (small, middle, large). All 3 dominant species were mainly found in chains or chain fragments in the samples, normally between 2 and 10 cells long. Around 50 units (chains, chain fragments or single cells) of each group or subgroup were counted. At least 100 individual cells were counted simultaneously. The dimensions of at least 20 cells of each group or subgroup were measured.

Determination of picocyanobacteria using flow-cytometry. Samples were fixed with a mixture of glutaraldehyde and paraformaldehyde (0.05 and 1% final concentration, respectively, see Jacquet et al. 1998a) for 15 min in dim light conditions, frozen in liquid nitrogen and kept at –80°C until flow-cytometry analysis was performed with a FACSort instrument (Becton Dickinson) as described in Marie et al. (1999, 2000). Picocyanobacteria were discriminated on the basis of

their forward- and right-angle light scatters (FALS and RALS), chl, and phycoerythrin fluorescence. Cellular parameters were normalised to the values measured for 0.95 µm beads. Data were collected in listmode files and then analysed using the CYTOWIN freeware of Vaulot (1989, available at http://www.sb-roscoff.fr/Phyto/cyto.html).

Statistical analyses. Data on biomass estimates of the different phytoplankton groups obtained by microscopy were compared to the relative contribution of the phytoplankton groups to the total chl *a* obtained by the CHEMTAX program using a Pearson product-moment correlation. In addition, microscopy data on picocyanobacteria were compared to those obtained from flow-cytometry, and total chl *a* measured by fluorometry was compared to chl *a*, and to chl *a* plus chlorophyllide *a* measured by HPLC using a Pearson product-moment correlation. All statistical analyses were performed using the software package SigmaStat.

RESULTS

Pigmented flagellate groups

The output ratios of pigments to total chl *a* from the CHEMTAX calculations are shown in Table 1, and on average varied 25% from the initial loaded ratios. We combined 2 types of prasinophytes and 2 types of haptophytes detected by the CHEMTAX program (see 'Materials and methods—Use of CHEMTAX program') into 1 group each (Fig. 2). In addition, 2 other pigmented flagellate groups were identified based on pigment analyses: dinoflagellates and cryptophytes (Fig. 2). Microscopy identified these 4 pigmented flagellate groups as well, and in addition 'other pigmented flagellates' belonging to other algal classes. The contribution of this last group to the total pigmented flagellate biomass was generally low and never exceeded 14% (Fig. 2).

Within each of the dominating pigmented flagellate groups (i.e. dinoflagellates, prasinophytes, haptophytes and cryptophytes), the correlations between data obtained by microscopy and by the CHEMTAX program were significantly positive using 55 data pairs from all 5 enclosures (Fig. 3). When data from the 5 enclosures were analysed separately, correlations for dinoflagellates, haptophytes and cryptophytes were significantly positive for 4 enclosures, while prasinophytes were significantly positive for 3 enclosures (Fig. 3).

The total chl *a*:carbon biomass ratios using all data were approximately 1:6 for dinoflagellates, between 1:5 and 1:15 for prasinophytes, between 1:10 and 1:25 for cryptophytes and between 1:2 and 1:4 for haptophytes (Fig. 3).

Cyanobacteria

The biomass of cyanobacteria (Fig. 2) was comprised of *Synechococcus*-like cells only. The development of cyanobacteria biomass obtained from microscopy was negatively correlated to that obtained from pigment analyses using all data (Fig. 4B). When data from the 5 enclosures were compared separately, no significant correlation was found for enclosures +NP and +NPSi: significantly negative correlations were found for enclosures +NPC and +NPCSi; while data from the enclosure without additions had a significantly positive correlation (Fig. 4B).

Data obtained by microscopy from 3 enclosures (+NP, +NPC and no addition) were compared to those obtained by flow-cytometry. In each case, correlations were significantly positive ($p < 0.05$) (Fig. 4C). Using data from all 3 enclosures the relationship between microscopy counts and flow-cytometry counts was close to 1:1 (Fig. 4C). Flow-cytometry data from enclosures NPSi and NPCSi were not available.

Table 1. Output ratios of pigments normalised to total chlorophyll *a* calculated by CHEMTAX

	Peridinin	19′-but	Fucoxanthin	Neoxanthin	19′-hex	Prasinoxanthin	Violaxanthin	Alloxanthin	Zeaxanthin	Chlorophyll *b*
Dinoflagellates	0.246									
Prasinophytes[a]				0.061		0.311			0.042	0.049
Prasinophytes[b]				0.031			0.078		0.024	0.301
Cryptophytes								0.288		
Haptophytes Type 3			0.206		0.228					
Haptophytes Type 4		0.060	0.209		0.090					
Diatoms			0.297							
Cyanobacteria									0.555	

[a]Prasinophytes with prasinoxanthin; [b]prasinophytes without prasinoxanthin

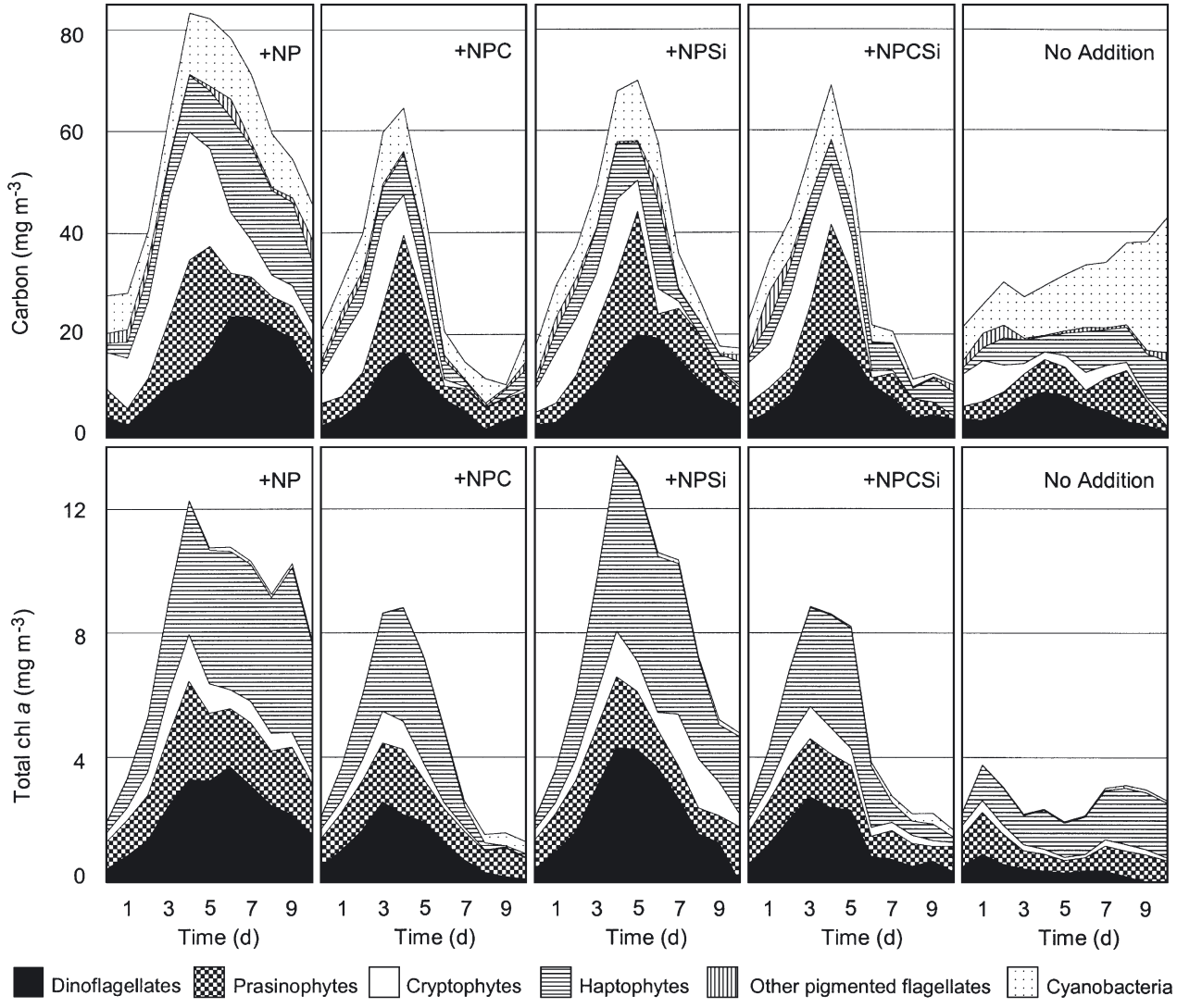


Fig. 2. Biomass of pigmented flagellates and cyanobacteria estimated by microscopy (top graphs) and by pigment analyses using CHEMTAX program (bottom graphs)

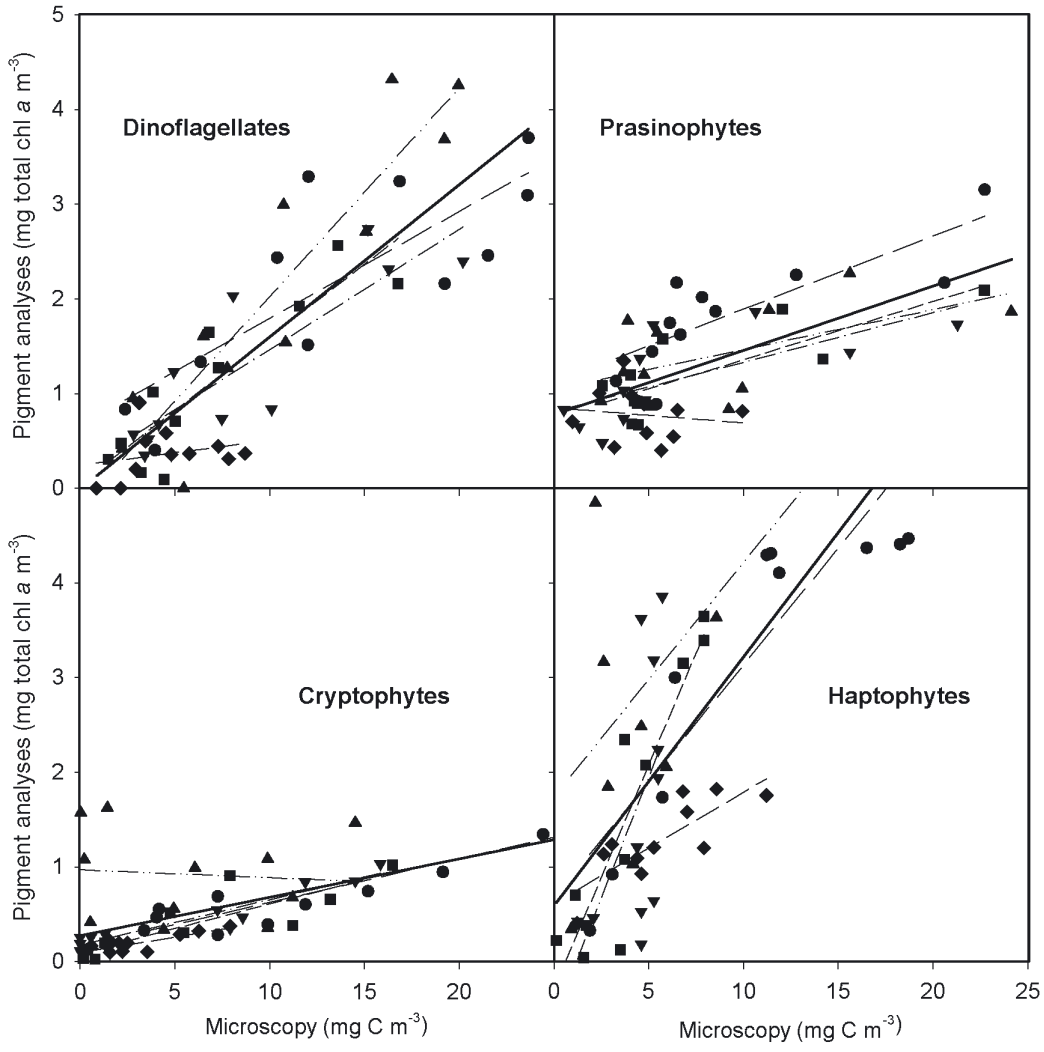
Diatoms

Diatom biomass was dominated by *Skeletonema costatum* [Greville] Cleve, *Dactyliosolen fragilissimus* [Bergon] Hasle comb. nov., and *Chaetoceros curvisetus* Cleve (Fig. 5), forming chains up to 1 mm in length. The development of diatom biomass obtained from microscopy was positively correlated to that obtained from pigment analyses in each of the 5 enclosures ($p < 0.05$) (Fig. 4A). The total chl *a*:carbon biomass ratio using all data was between 1:50 and 1:100 (Fig. 4A).

DISCUSSION

Data obtained by microscopy showed significantly positive correlations with those obtained by the CHEMTAX program for the development of dinoflagellates, prasinophytes, cryptophytes, haptophytes, and diatoms (Figs. 3 & 4A). However, when comparing biomass estimates made by pigment analysis and CHEMTAX with estimates made by microscopy, we found a large variation in total chl *a*:carbon ratios amongst the different phytoplankton groups. This was probably due to differential over- and under-estimations by the 2 methods.

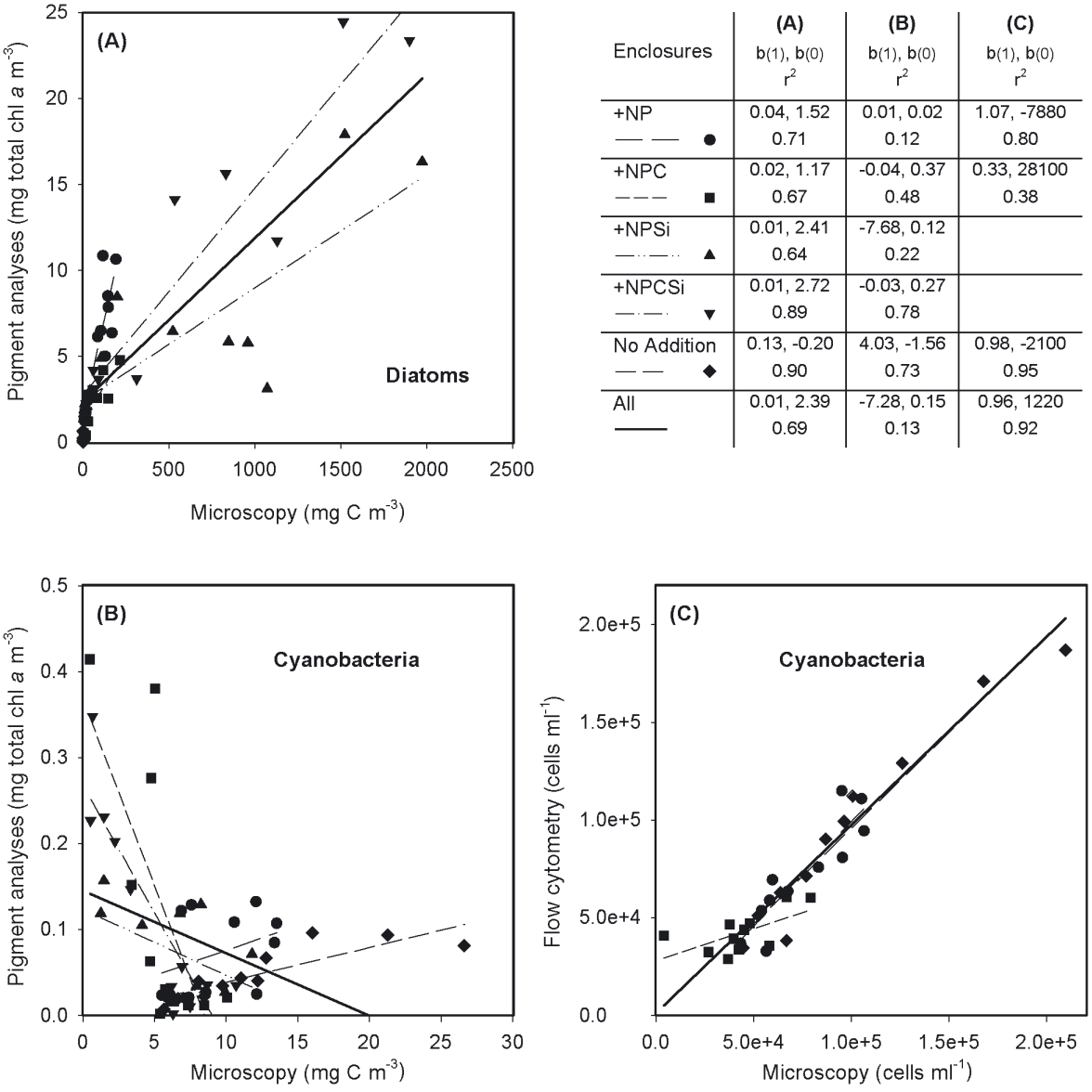
Estimated diatom contribution to total phytoplankton biomass was higher when based on microscopy than when based on pigment analyses, especially in the Si-amended enclosures (Figs. 2 & 5). Total chl *a*:carbon ratios for diatoms were between 1:50 and 1:100 (Fig. 4A) and thus at the lower end of the total chl *a*:carbon conversion factors, that often lie between 1:27 and 1:67 for natural phytoplankton populations



Enclosures	Dinoflagellates b(1), b(0), r^2	Prasinophytes b(1), b(0), r^2	Cryptophytes b(1), b(0), r^2	Haptophytes b(1), b(0), r^2
+NP	0.11, 0.67, 0.64	0.08, 1.12, 0.66	0.05, 0.16, 0.84	0.25, 0.66, 0.82
+NPC	0.15, 0.05, 0.80	0.06, 0.74, 0.65	0.05, 0.09, 0.74	0.47, -0.29, 0.84
+NPSi	0.22, -0.19, 0.84	0.04, 1.04, 0.31	-0.01, 0.97, 0.01	0.25, 1.74, 0.38
+NPCSi	0.13, 0.19, 0.74	0.05, 0.81, 0.49	0.05, 0.18, 0.90	0.51, 0.61, 0.29
No Addition	0.03, 0.24, 0.06	-0.02, 0.85, 0.02	0.03, 0.08, 0.67	0.12, 0.62, 0.65
All	0.16, -6.33, 0.73	0.07, 0.77, 0.46	0.04, 0.27, 0.36	0.26, 0.59, 0.52

Fig. 3. Pigmented flagellate biomass estimated by microscopy in relation to biomass estimated by pigment analyses using CHEMTAX program. Table below figure shows symbols for enclosures and linear regression coefficients

(Riemann et al. 1989). It is unlikely that diatom carbon had been overestimated by microscopy, since we assumed a plasma layer thickness of 1 µm surrounding the vacuole (Strathmann 1967). If we had assumed a plasma layer thickness of 2 µm (Hitchcock 1983, Hillebrand et al. 1999), the diatom biomass estimated by microscopy would have been 1.5 times higher, resulting in a further decrease in the total chl *a*:carbon ratio. It seems more likely that the low total chl *a*:carbon ratio found during the last 5 d of this study, especially in the Si-amended enclosures (Figs. 4A & 5), was caused by the decrease in pigment content in diatom cells reach-



Enclosures	(A) b(1), b(0) r^2	(B) b(1), b(0) r^2	(C) b(1), b(0) r^2
+NP ●— —	0.04, 1.52 0.71	0.01, 0.02 0.12	1.07, -7880 0.80
+NPC ■— — —	0.02, 1.17 0.67	-0.04, 0.37 0.48	0.33, 28100 0.38
+NPSi ▲— ·— ·	0.01, 2.41 0.64	-7.68, 0.12 0.22	
+NPCSi ▼— · — ·	0.01, 2.72 0.89	-0.03, 0.27 0.78	
No Addition ◆— —	0.13, -0.20 0.90	4.03, -1.56 0.73	0.98, -2100 0.95
All ——	0.01, 2.39 0.69	-7.28, 0.15 0.13	0.96, 1220 0.92

Fig. 4. (A) Diatom and (B) cyanobacteria biomass estimated by microscopy in relation to biomass estimated by pigment analyses using CHEMTAX program. (C) Cyanobacteria density estimated by microscopy in relation to estimations by flow-cytometry using data from enclosures +NP, +NPC, and no addition. No samples for flow-cytometry were taken from enclosures +NPSi and +NPCSi. Table on upper right shows symbols for the enclosures and linear regression coefficients

ing the late growth phase, resulting in a decrease in the chl *a*:carbon ratio from 1:50 to 1:100, as reported by Llewellyn & Gibb (2000). In addition, the diatoms that were blooming in the Si-amended enclosures during that period were relatively large cells (Fig. 5). Large diatoms have a lower chl *a*:carbon ratio than small diatoms according to Breton et al. (2000), who made estimates from data published by Stauber & Jeffrey (1988) regarding 51 species of marine diatoms. The smallest diatoms had a chl *a* + *c* content of 10^4 fg μm^{-3}, while the largest diatoms had a chl *a* + *c* content of only 45 fg μm^{-3}.

Total chl *a*:carbon ratios for pigmented flagellate groups were all above 1:27. Changes in nutrient and light regimes may explain some variability in the total chl *a*:carbon ratio (Sciandra et al. 2000), such as those recorded in the present study for cryptophytes (between 1:10 and 1:25; Fig. 5). For the other pigmented flagellate groups (dinoflagellates, prasinophytes and haptophytes), however, total chl *a*:carbon ratios between 1:2 and 1:15 (Fig. 3) rather support an overestimation of their contribution to the total chl *a* by the CHEMTAX program, or the high total chl *a*:carbon ratios were partly caused by an underestimation of the

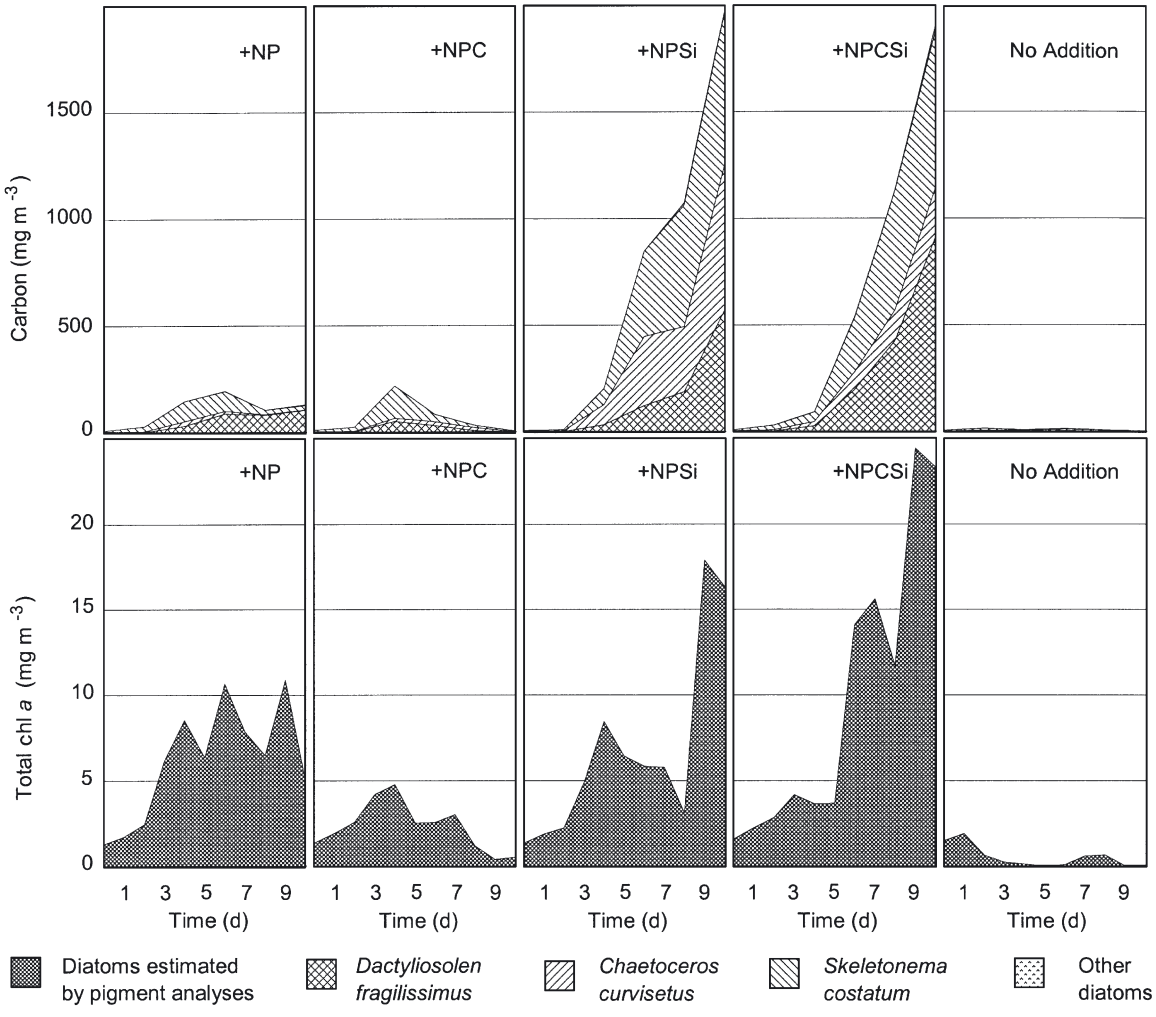


Fig. 5. Diatom biomass estimated by microscopy (top graphs) and by pigment analyses using CHEMTAX program (bottom graphs)

biomass of pigmented flagellates by microscopy. In the present investigation we multiplied the linear dimensions of naked flagellates by 1.1 to compensate for shrinkage due to fixation (Choi & Stoecker 1989), and converted the resulting plasma volume to carbon using the factor 0.11 pg μm^{-3} (Strathmann 1967). In contrast, Børsheim & Bratbak (1987) recommended a conversion factor of 0.22 pg μm^{-3} for glutaraldehyde-fixed cells of naked flagellates, which would result in a 50% higher estimate of carbon biomass for pigmented flagellates in the present investigation.

While the trends in the development of pigmented flagellates and diatoms were significantly positively correlated between pigment analyses and microscopy in the present investigation, there was no significantly positive correlation for the cyanobacteria (Fig. 4B). The CHEMTAX program underestimated the amount of cyanobacteria considerably (Figs. 2 & 4B). This can be seen by comparison of the microscopy data with data from flow-cytometry performed on the same water samples, which revealed an almost identical abundance of cyanobacteria (Fig. 4C). Flow-cytometry has previously been shown to provide excellent counting statistics for picocyanobacteria (Li & Wood 1988, Jacquet et al. 1998b). In both, flow-cytometry and epifluorescence microscopy, picocyanobacteria were identified by their size and shape and by the orange autofluorescence of phycoerythrin in blue light (Marie et al. 2000). In contrast, pigment analyses do not use phycoerythrin, but zeaxantin, as the marker pigment for cyanobacteria (Millie et al. 1993). Zeaxanthin is known not to covariate with the chl *a* content (Liu et al. 1999), and the zeaxanthin:chl *a* ratio in cyanobacteria has been shown to vary between 0.4 and 2, depending on exposure to different irradiances (Kana et al. 1988, Mackey et al. 1998, Schlüter et al. 2000). In this study the zeaxanthin concentrations were very low, and even though the CHEMTAX program resulted in a rel-

atively low normalised zeaxanthin:total chl *a* ratio of 0.55 (Table 1), the cyanobacteria biomass determined by this means was extremely small compared to the results of microscopy and flow-cytometry, indicating that the zeaxanthin content of the *Synechococcus*-like population in this brackish environment was probably negligible. Microscopic counts of cyanobacteria and CHEMTAX calculations of cyanobacteria biomass have previously shown significantly positive correlation for samples taken from the northern part of the Australian sector of the Southern Ocean, whereas no cyanobacteria were detected by microscopy in samples taken from the southern part, for which CHEMTAX computed small amounts (Wright et al. 1996). More culture studies should be conducted to determine the pigment content of different strains of *Synechococcus*, preferably on strains isolated from coastal areas.

Assessing 90% of the phytoplankton biomass in the microscope, as recommended by Willén (1976), would not have revealed the presence of phytoplankton groups other than diatoms in the Si-enriched enclosures during the last 5 d of the experiment (Figs. 2 & 5). In the present investigation, however, an extra effort has been made to analyse all groups recorded by pigment analyses. Using epifluorescence microscopy, up to 10 subsamples of the same water sample have been assessed in order to get statistically significant estimates of phytoplankton groups accounting for <10% of the phytoplankton biomass; this would not have been practically feasible with a routine monitoring programme. Both microscopy and pigment analyses revealed that species belonging to dinoflagellates, prasinophytes, cryptophytes, and haptophytes had a biomass in the Si-enriched enclosures that was similar to that observed in the Si-depleted enclosures, where these groups accounted for a substantial part, i.e. >30% of the phytoplankton biomass (Figs. 2 & 5). This fact is important, because an analysis based on microscopy assessing 90% of the phytoplankton biomass would have characterised samples taken from the Si-enriched enclosures during the last 5 d of the investigation period as environments with low phytoplankton diversity, with only 3 diatom species present (Willén 1976, Margalef 1978). In contrast, both epifluorescence microscopy and pigment analyses using the CHEMTAX program revealed the presence of at least 4 additional algal classes, making up a 'background population' in the diatom-dominated environments that was similar to that in the enclosures without silicate enrichment (Figs. 2 & 5).

Epifluorescence microscopy is a technical possibility, but its practical feasibility is very limited because assessment of a background population during a bloom of a single or a few species is extremely time-consuming. The water samples have to be divided into many subsamples and large filter areas have to be screened.

Pigment analyses using the CHEMTAX program also characterise the diversity of a phytoplankton community at group level. In addition, HPLC pigment analysis offers high reproducibility (Schlüter et al. 2000). A weak side of the method in the present study was its failure with regard to cyanobacteria, but picocyanobacteria can readily be assessed using flow-cytometry. Pigment analyses without identifying the dominant and subdominant species in the microscope, however, can result in a completely wrong picture of the phytoplankton community. For instance, *Prymnesium parvum*, a common haptophyte causing fish-kills, only contains traces of 19′-hexanoyloxyfucoxanthin, the marker pigment for haptophytes (Jeffrey & Wright 1994), and on the basis of its fucoxanthin content it would be classified erroneously as a diatom. When applying HPLC pigment analyses and flow-cytometry in routine monitoring we therefore recommend screening the sample by microscopy for dominant and subdominant species.

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