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Research Article

Effect of nutrients on culture dynamics of marine phytoplankton

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Abstract. The effects of nitrate, ammonium and phosphate on the abundance, chlorophyll *a* content (chl *a*), *in vivo* fluorescence, particulate organic carbon, nitrogen, phosphorus and cell morphology of the diatom *Skeletonema costatum*, the dinoflagellate *Prorocentrum micans* and the coccolithophore *Emiliania huxleyi* were investigated in the laboratory. The carbon:chlorophyll *a* ratio (as weight), a parameter often used in productivity estimates, differed substantially among the three species as well as at different nutrient concentrations at the end of the exponential growth phase. The cell chl *a* content was higher in the earlier phases of growth in all three species in agreement with previous investigations. Average chl *a* content per cell during the experiments was higher in *P. micans* (8 pg) than in *S. costatum* (0.4 pg) and *E. huxleyi* (0.2 pg). However, chl *a* content per unit volume was higher in *E. huxleyi* (~15 fg μm^{-3}) than in *S. costatum* (~7 fg μm^{-3}) and *P. micans* (~1 fg μm^{-3}). *Prorocentrum micans* cultures reached the highest total biovolume (74 mm^3 L^{-1} and was 3 and 5 times higher than *S. costatum* and *E. huxleyi* cultures, respectively) under high nutrient concentrations. Nevertheless, total chl *a* concentration of *S. costatum* culture was almost twice higher (122 µg L^{-1}) than that of the other two species at the end of the exponential growth phase. Phosphate consumption by *S. costatum* occurred at higher rates compared to the other two species during the experimental period, probably showing that this nutrient was more favourable for this species. Our findings from these laboratory experiments emphasize that chl *a* values in the natural habitat may not accurately indicate actual phytoplankton biomass.

Key words. Phytoplankton cultures; chlorophyll *a;* carbon, biovolume; *in vivo* fluorescence.

Introduction

Phytoplankton is of utmost importance in global carbon dynamics (Geider et al., 2001). The diatom *Skeletonema costatum* (Grev.) Cleve, the coccolithophorid *Emiliania huxleyi* Lohmann and the dinoflagellate *Prorocentrum micans* Ehrenberg are among the most widespread phytoplankton species in the World's oceans during bloom periods (Fukuyo et al., 1990; Han et al., 1992; Brown and Yoder, 1994). Among these species *E. huxleyi* was recognized as a significant species in recent years for its role in climate change (Holligan et al., 1983; Charlson et al., 1987; Taylor et al., 1990; Balch et al., 1991; Brown and Yoder, 1994; Anning et al., 1996; Çokacar et al., 2001).

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Its large-scale blooms can be distinguished in visible satellite imagery because of their high reflectance property in the surface layer (Balch et al., 1991). *Skeletonema costatum* is a cosmopolitan diatom species (Tomas, 1997) and often dominates the spring bloom in coastal waters (Han et al., 1992; Thornton and Thake, 1998). *Prorocentrum micans* is one of the most common dinoflagellate species in cold temperate to tropical waters. *Prorocentrum micans* forms extensive red tides in many parts of the World`s oceans (Fukuyo et al., 1990) and sometimes co-occurs with the toxic dinoflagellate *Alexandrium* species in natural blooms (Cannon, 1990; Esteves et al., 1992). These latter two species are mainly neritic and estuarine, but they also are found in oceanic environments (Tomas, 1997) and could be important in terms of total carbon mass when they reach bloom concentrations.

Nutrients are among the most important factors controlling phytoplankton growth. However, nutrient contents in marine environments greatly change over space and time. Variations in phytoplankton biomass, greatly influenced by nutrients, are often monitored by means of chl *a* concentrations (Banse, 1977) from either *in situ* or remote sensing data (Gitelson et al., 1996; Stumpf, 2001; Gohin et al., 2003; Richardson et al., 2004; Greenan et al., 2004). Consequently, estimates related with current environmental issues like oceanic primary production, CO_2 drawdown, dimethyl sulphide (DMS) production by algae and global climate change are based on chl *a* concentrations detected with *in situ* sampling or from satellites (Banse, 1977; Balch et al., 1992; Brown and Yoder, 1994; Longhurst et al., 1995; Field et al., 1998; Joint and Groom, 2000; Geider et al., 2001). Satellite data, in particular, generally become insufficient to observe changes in phytoplankton species composition and therefore may not accurately indicate phytoplankton biomass since chl *a* content of phytoplankton varies from species to species. Additionally, changes in nutrient content in the environment may have profound effects on chl *a* and carbon dynamics within each phytoplankton cell. Thus, mechanisms controlling phytoplankton growth and physiology should be taken into account in assessments of nutrient cycling, carbon and primary production. Despite the enormous amount of work on the effects of different kinds of nutrients (and light) on bloom dynamics, cell physiology, morphology, life cycle, biogeochemistry, DMS production, CO_2 release, carbon flux and grazers of some phytoplankton species like *E. huxleyi*, there is limited information from the laboratory on the effects of nutrients on chl *a* and carbon dynamics of other phytoplankton species and even less information from comparative studies using different species (Harrison et al., 1977; Cullen, 1982; Goldman, 1980). It was reported that C:chl *a* ratio may be as low as 10 (Laws and Bannister, 1980) or as high as 250 or more (Holmes et al., 1967; Banse, 1977; Laws and Bannister, 1980; Cullen, 1982) in natural phytoplankton, and therefore biomass or production values calculated based on carbon and chlorophyll are quite rough estimations. This variation may be the reason for the discrepancy observed between phytoplankton biomass and chl *a* concentration in some field investigations (Eker-Develi, 2004; Ediger et al., accepted). Apparently, there is a need for more data to approximate algal biomass from chl *a* measurements.

In the present study, we compared the effects of nitrate, ammonium and phosphate on bloom characteristics (cell abundance and biomass), *in vivo* fluorescence, chl *a* content and cell morphology of phytoplankton species representative of the most common groups of phytoplankton. Although these studies were performed in the laboratory with batch cultures during various growth phases, the reported results could provide important insights for evaluating phytoplankton dynamics in nature.

Material and methods

Ply # 92 E strain of *Emiliania huxleyi* (scale bearing S-cells), Ply # 95A strain of *Prorocentrum micans* and Ply #106 strain of *Skeletonema costatum* were obtained from Plymouth Marine Laboratory (Plymouth, United Kingdom). Stock cultures were grown in 0.250-L glass borosilicate flasks containing F/2 medium (880 µM nitrate, 36 µM phosphate) at 20 ± 2 ºC, with 100 µmol m^{-2} s^{-1} irradiance and 12-h light, 12-h dark cycle. Seawater collected from ~8 nautical miles offshore (from the Institute of Marine Sciences, Erdemli, Mediterranean coasts of Turkey) was filtered (0.45 µm-porosity cellulose acetate filters) and autoclaved prior to the experiments. In the experiments, batch cultures of triplicate or quadruplet samples were assessed for *in vivo* fluorescence measurements using generally two 0.250-L (which take up little space on the shelves of the temperature controlled room) and two 1-L borosilicate flasks (from which nutrient and chl *a* samples were taken). Statistical analysis showed no difference ($p > 0.05$ single factor ANOVA) between replicates. The light source was below the shelves on which the Erlenmeyer flasks (having large bottom areas) were placed, and the light availability should be similar for all flasks. Nitrate and phosphate concentrations were reduced to 10 % of the F/2 medium for each experimental set (Table 1). For ammonium treatment, ammonium substituted for nitrate at 5, 50, or 880 µM (*S. costatum*, *P. micans*), or 5 or 50 µM ammonium (*E. huxleyi*). Samples of *S. costatum*, *E. huxleyi* and *P. micans* were taken at 1, 2 and 3 d intervals, respectively, between the second and third h of the light period during each experiment. Initial cell concentrations after inoculation were 1×10^6 cells L^{-1}, 3×10^6 cells L^{-1}, 0.05×10^6 cells L^{-1} for *E. huxleyi*, *S.*

costatum and *P. micans,* respectively. Cell counts (~100–500 cells depending on culture density) were performed by using a Sedgewick Rafter Cell under a phase contrast inverted microscope at 200 × magnification. Growth rates were determined by regressions of ln cell number vs. time during exponential phase using the formula; $\ln N_t - \ln N_0 = \mu(t - t_0)$ where N_t is the cell number at time t , N_0 is the cell number at time 0 (t_0), and μ is the growth rate.

The volume of each cell during the late exponential phase was calculated by measuring morphometric characteristics (diameter, length, width; n = 100 cells as in Menden-Deuer and Lessard, 2000, Hillebrand et al., 1999). Assuming a cylindrical shape, biovolume of *S. costatum* (for the size 4 × 5 μm) was calculated as 62 μm^3 and biovolume of *P. micans* as 7400 μm^3 (for the width = W, length = L and height = H, 31, 38, 15 μm, respectively) when its shape was assumed to be 0.8 times oval (volume = 4/3 × 3.14 × W/2 × L/2 × H/2 multiplied by 0.8 since this species is almond shaped, Hillebrand et al., 1999; Menden-Deuer and Lessard, 2000). Average biovolume of *P. micans* increased to 14,600 μm^3 under phosphate limitation. *Emiliania huxleyi* biovolume was calculated as 14 μm^3 from the sphere volume when cell diameter was 3 μm.

In vivo fluorescence was measured in a 3.5 ml quartz cell using a Hitachi F-3000 spectrofluorimeter (Japan).

The carbon and nitrogen contents of *E. huxleyi*, *S. costatum* and *P. micans* at the end of the exponential growth phase (i. e. when cells reached highest abundances, 15th, 7th and 23rd d; Figs. 1, 2, 3) were measured using a Carlo Erba 1108 CHN analyser (Italy). All filters were frozen (–20 °C) immediately after sampling. Within a few weeks, they were dried at 50–60 °C overnight and stored in a vacuum desiccator until analysis. The particulate organic carbon (POC) and particulate organic nitrogen (PON) samples were exposed to hydrochloric acid fumes, re-dried as above, and analysed the next day by the dry combustion technique of Polat and Tugrul (1995). Particulate phosphorus (PP) samples were frozen similar to POC and PON immediatelly after sampling and within a few weeks combusted at 500 °C for 3 h, treated with 12 ml of 0.5 N HCl and filtered. After adjusting the pH to 8, the total phosphorus in the solution was determined colorimetrically (Polat and Tugrul, 1995). PP content of *E. huxleyi* was not measured. Unfortunatelly, no replicates were taken for POC, PON and PP samples.

For chl *a* analyses, 10–20 ml samples were filtered through 0.7 μm porosity GF/F filters and kept frozen at –20 °C (Mantoura et al., 1997) until analysis within 1 or 2 months. Samples were extracted in 90 % acetone and held at 4 °C overnight in darkness. Absorbance was measured using a Hitachi F-3000 spectrofluorimeter (detection limit, 0.01 μg L^{-1}) and calibration was performed using a commercially available chl *a* standard from Sigma-Inc., St. Louis, MO, USA. (Strickland and Parsons, 1972; Holm-Hansen and Riegman, 1978).

Table 1. Nutrient concentrations (μM) for the experimental sets of *Skeletonema costatum*, *Prorocentrum micans* and *Emiliania huxleyi.*

NO_3-N	PO_4-P	NH_4-N	N:P (molar)	Medium
880	36		24	F/2
88	36		2.4	NO_3^- reduced F/2
880	3.6		240	PO_4^{3-} reduced F/2
	36	*880	24	F/2-NO_3^- + 880 μM NH_4^+
	36	50	1.4	F/2-NO_3^- + 50 μM NH_4^+
	36	5	0.14	F/2-NO_3^- + 5 μM NH_4^+

* This ammonium concentration was not used in *Emiliania huxleyi* cultures.

Samples of oxidized nitrogen ($NO_3 + NO_2$-N, hereafter referred to as N), ortho-phosphate (PO_4^{-3}-P, referred to as P) and ammonium (NH_4-N) were collected in acid-cleaned high density polyethylene bottles (first washed in 10 % HCl and then rinsed thoroughly with distilled water prior to sampling) and held frozen (–20 °C) until analysis within 2 months. Standard colorimetric methods were applied (Strickland and Parsons, 1972; Grasshoff et al., 1983) using a Technicon model 3-channel Autoanalyzer (Bran & Luebbe, Norderstedt, Germany). The detection limits for nitrate, phosphate and ammonium were 0.05 μM, 0.02 μM and 0.2 μM, respectively.

Results

Abundance and growth rates

In F/2 medium, *E. huxleyi* reached the highest abundance (~1,200 × 10^6 cells L^{-1}) and *S. costatum* and *P. micans* followed this species (~350 × 10^6 cells $L^{-1}$and 10 × 10^6 cells L^{-1}, respectively) (Figs. 1, 2, 3).

Skeletonema costatum attained the highest growth rate (0.7–0.8 day^{-1}), and *E. huxleyi* and *P. micans* followed this species (0.5 d^{-1} and 0.2 d^{-1} respectively) in F/2 medium (Table 2).

Except *S. costatum* grown in nitrate-reduced cultures, a 10 % reduction in nutrients caused a decrease in abundance, chl *a* and *in vivo* fluorescence for the species investigated (Figs. 1, 2, 3, Table 2). When nitrate concentrations were reduced to 10 % of F/2 medium, phosphate along with nitrate was consumed almost completely in the *S. costatum* culture (Fig. 1b). In contrast, when nitrate was reduced for the other two species, this nutrient was almost completely diminished while phosphate was still available in sufficient amounts (Figs. 2b, 3b).

The duration of the *E. huxleyi* experiment was probably too short to observe all the growth phases. There were still high amounts of nitrate and phosphate at the end of the experiment on day 17 in F/2 medium. About

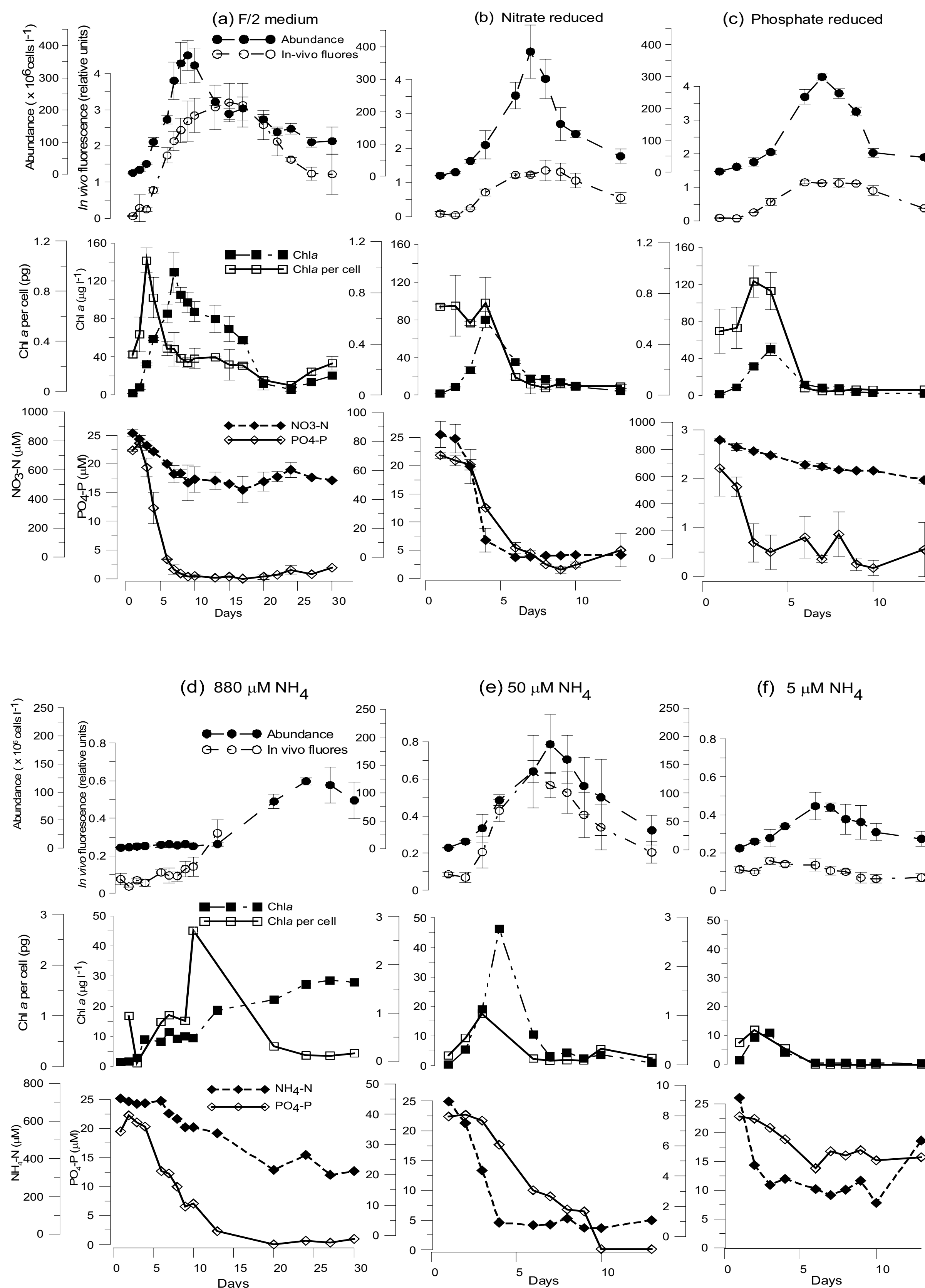

Figure 1. Variations in abundance, in vivo fluorescence and chl *a* concentrations at different nutrient treatments in *Skeletonema costatum* (standard deviations that are not visible in b) and c) show low deviations). See Table 1 for nutrient medium used.

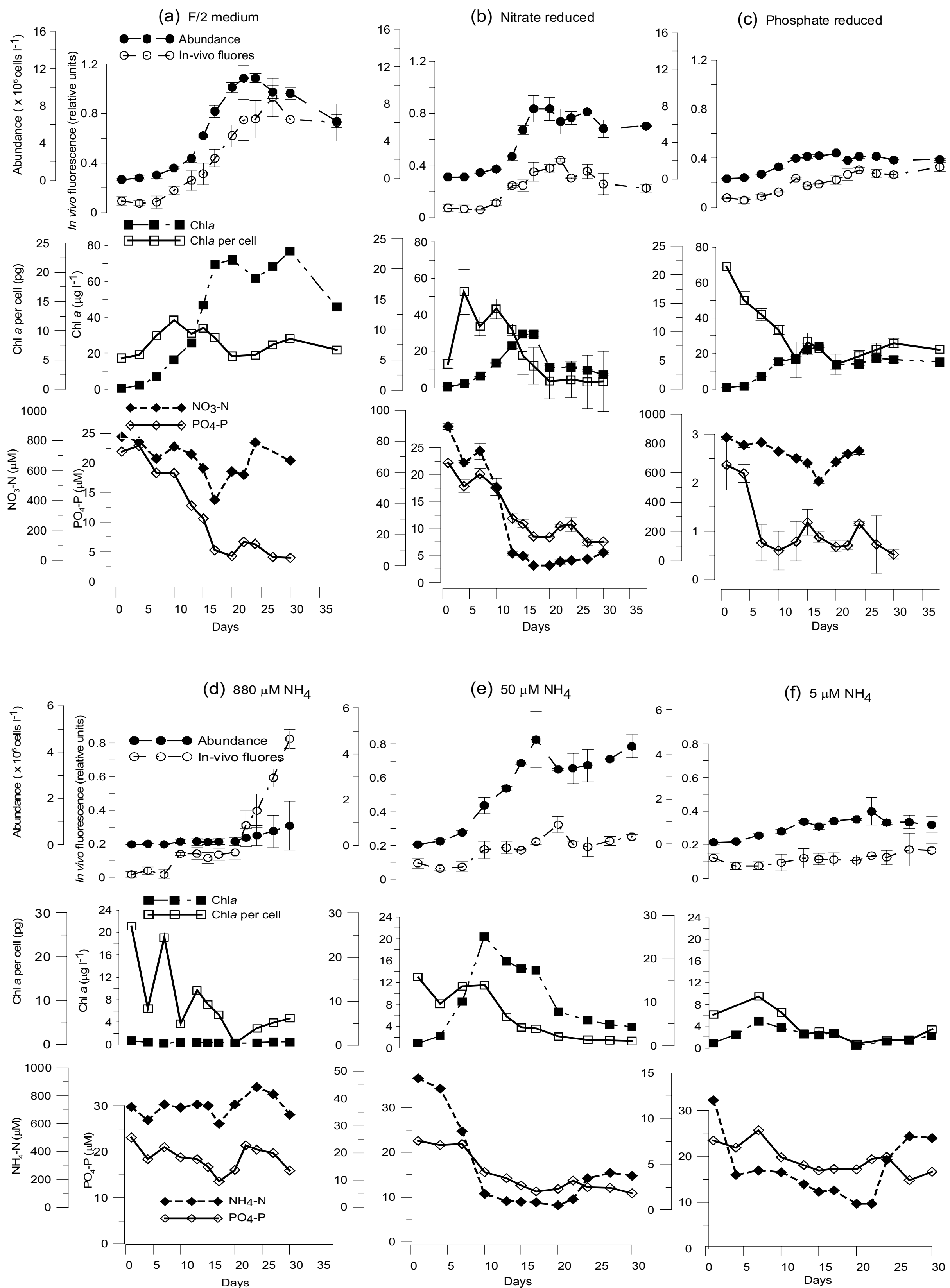

Figure 2. Variations in abundance, in vivo fluorescence and chl *a* concentrations at different nutrient treatments in *Prorocentrum micans* (standard deviations that are not visible in b) and c) show low deviations). See Table 1 for nutrient medium used.

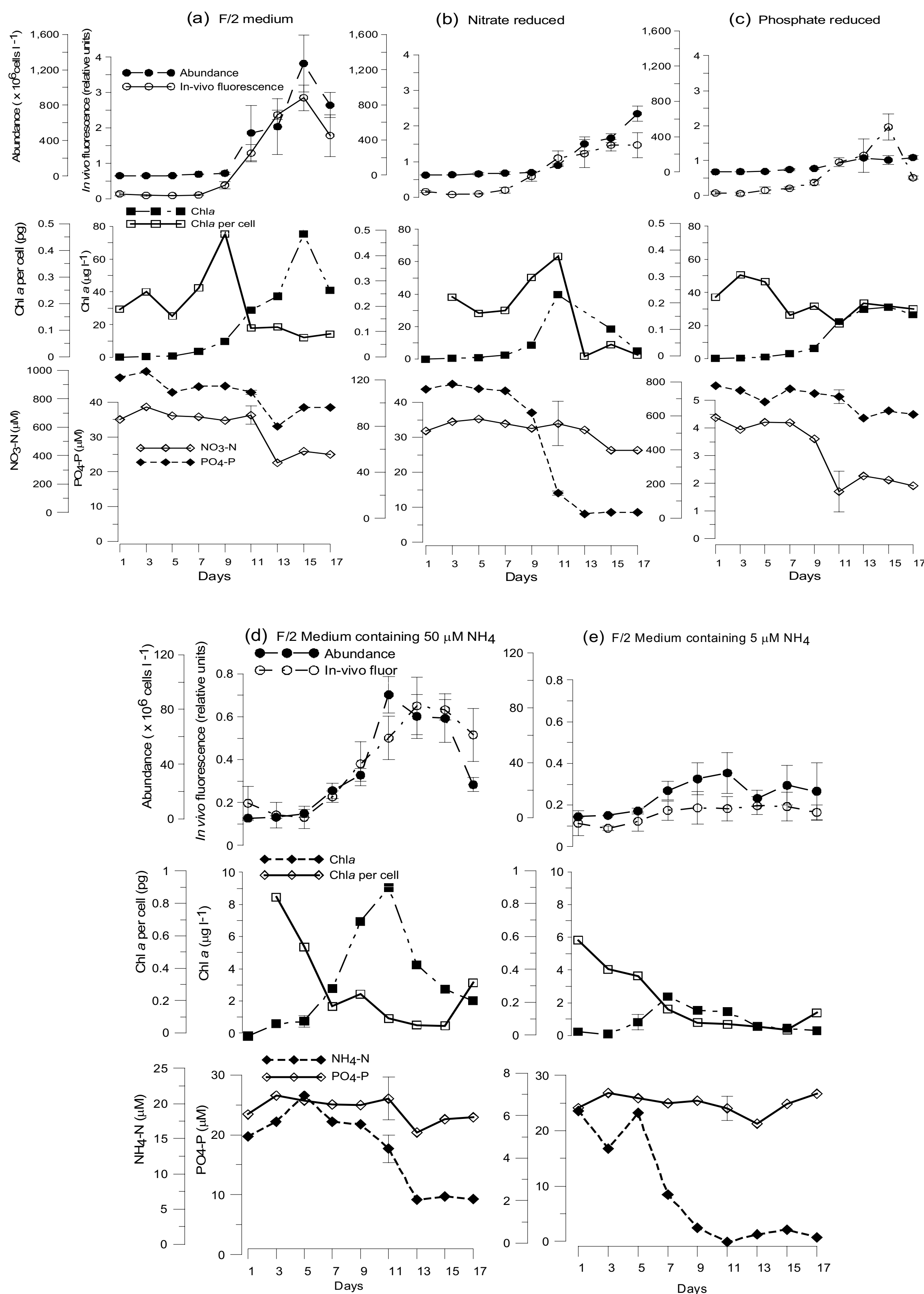

Figure 3. Variations in abundance, in vivo fluorescence and chl *a* concentrations at different nutrient treatments in *Emiliania huxleyi* (replicates for chl *a* and nutrients were only for the 5th and 11th days, respectively). See Table 1 for nutrient medium used.

Table 2. Physiological parameters in three phytoplankton species (until the stationary phase, ± confidence limits, unfortunately there are no replicates in carbon, nitrogen and phosphorus measurements and some of the chl *a* samples) (a) *Skeletonema costatum*.

Skeletonema costatum	F/2	NO_3^- reduced	PO_4^{3-} reduced	880 µM NH_4^+	50 µM NH_4^+	5 µM NH_4^+
Growth rate*	0.76 ± 0.15	0.84 ± 0.18	0.79 ± 0.02	0.35 ± 1.6	0.77 ± 0.23	0.53 ± 1.6
Average fg chl *a* per µm^3 cell volume	7 ± 1	8 ± 1	8 ± 15	12	5	5
Total chl *a* concentration at the late exponential phase (µg L^{-1})	122 ± 49	80 ± 74	50 ± 59	30	46	11
Total biovolume of cells at the exponential phase (mm^3 L^{-1})	23 ± 25	24 ± 46	19 ± 5	0.7	14	7
Carbon content per cell (pg) on 7th day	40	33	27	na	24	na
C:chl *a* (pg:pg) on 7th day	91	1021	820	na	1749	na
Molar C:N (in cells) on 7th day	7.1	17.2	11.6	na	13.3	na
Molar C:P (in cells) on 7th day	~55	66.1	259.3	na	28.7	na
Molar N:P (in cells) on 7th day	~7	3.8	22.3	na	2.2	na

Cell volume was 62 µm^3 in F/2 medium.

Table 2. (b) *Prorocentrum micans.*

Prorocentrum micans	F/2	NO_3^- reduced	PO_4^{3-} reduced	880 µM NH_4^+	50 µM NH_4^+	5 µM NH_4^+
Growth rate*	0.20 ± 0.13	0.21 ± 0.06	0.21 ±0.05	0.12 ± 0.79	0.19 ± 0.09	0.09 ± 0.04
Average fg chl *a* per µm^3 cell volume	1.09	0.88± 0.87	0.82 ± 0.51	0.88	0.89	0.57
Total chl *a* concentration at the late exponential phase (µg L^{-1})	76	29 ± 5	18 ± 3	0.7	20	5
Total biovolume of cells at the exponential phase (mm^3 L^{-1})	74	45±87	21 ± 9	0.3	27	8
Carbon content per cell (pg) at 24th day	2110	4120	9041	na	6150	na
C:chl *a* (pg:pg) on 24th day	365	2160	1792	na	4800	na
Molar C:N (in cells) on 24th day	7.9	23.6	20.7	na	22.0	na
Molar C:P (in cells) on 24th day	68.0	103.7	486.9	na	128.5	na
Molar N:P (in cells) on 24th day	8.7	4.4	23.6	na	5.8	na

Cell volume was 7401 µm^3 in F/2 medium.

Table 2. (c) *Emiliania huxleyi.*

Emiliania huxleyi	F/2	NO_3^- reduced	PO_4^{3-} reduced	880 µM NH_4^+	50 µM NH_4^+	5 µM NH_4^+
Growth rate*	0.53 ± 0.05	0.63 ± 0.09	0.49 ± 0.02	na	0.51 ± 0.43	0.33 ± 0.56
Average fg chl *a* per µm^3 cell volume	15	12	15	na	20	12
Total chl *a* concentration at the late exponential phase (µg L^{-1})	75	40	31	na	9	2
Total biovolume of cells at the exponential phase (mm^3 L^{-1})	15	9	2.6	na	1.4	0.3
Carbon content per cell (pg) on 15th day	9.4	24.9	55.8	na	na	na
C:chl *a* (pg:pg) on 15th day	49	146	266	na	na	na
Molar C:N (in cells) on 15th day	7.2	12.6	8.2	na	na	na

* growth rate calculated from average abundance of two replicates.
na (not available)
Cell volume was 14 µm^3 in F/2 medium.

3 months following the termination of the *E. huxleyi* experiment (in F/2 medium) at high cell density (310 × 10^6 cells L^{-1}), plenty of phosphate (7.28 µM) was present in the flask, but nitrate decreased to a fraction (0.8 µM).

At 880 µM concentration, ammonium impeded the growth of both *S. costatum* and *P. micans*. Following an apparent adaptation period, *S. costatum* could begin to grow after 15 days. The highest ammonium concentration used for *E. huxleyi* was 50 µM, thus the effect of high ammonium concentration on this species could not be observed.

Total chlorophyll *a*

The culture reaching the highest chl *a* concentration was that of *S. costatum* in F/2 medium. Chl *a* concentration of this culture (122 µg L^{-1}) was almost twice that of *E. huxleyi* (75 µg L^{-1}) and *P. micans* (76 µg L^{-1}).

Excluding cultures with high ammonium, which became toxic for these species, when nutrient concentrations were reduced, total chl *a* concentration at the late exponential phase also decreased between 35 % and 97 %.

In vivo fluorescence:chlorophyll *a*

In vivo fluorescence per chl *a* (an estimate of inefficiency of photosynthetic electron transport, Lawlor, 1987; Muggli and Harrison, 1997) was higher in nutrient-limited cultures than in high-nutrient cultures during the exponential growth phase (Fig. 4). This ratio was highest under low ammonium availability in all three cultures (excluding *P. micans* cultures with high ammonium content 880 µM, Fig. 4). In *S. costatum* cultures, this ratio was higher under P limitation compared to N limitation. However, there was not much difference in this ratio under P or N limitation or high nutrient conditions in *P. micans* and *E. huxleyi* cultures.

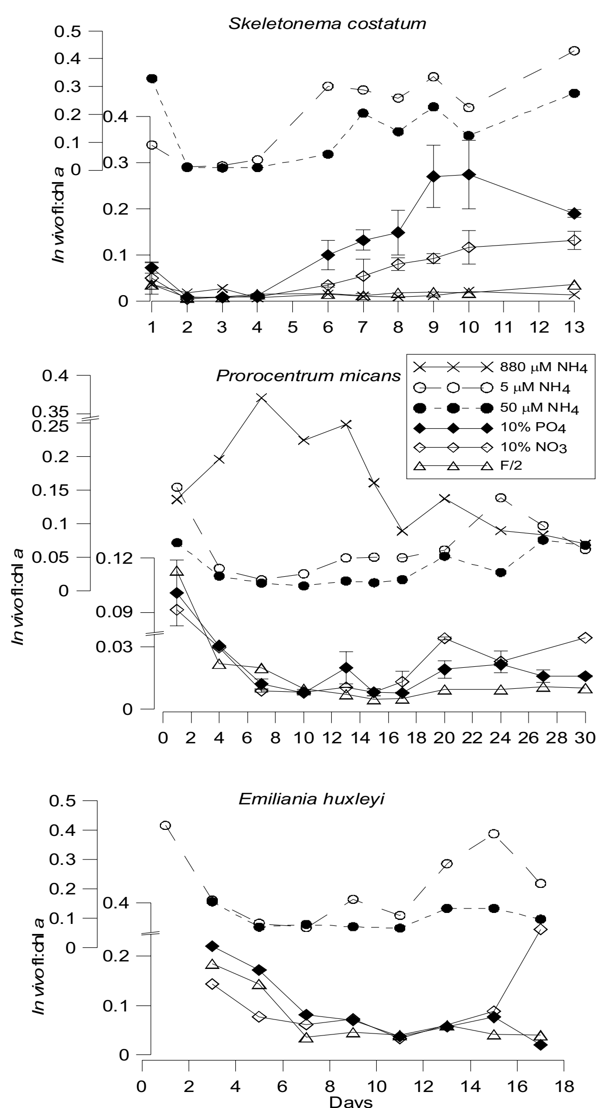

Figure 4. In vivo fluorescence:chl *a* ratios in *Skeletonema costatum*, *Prorocentrum micans* and *Emiliania huxleyi*.

Cellular C:N:P ratios

Under high nutrient conditions and at the end of the exponential phase, molecular C:N:P ratios of *S. costatum* and *P. micans* were ~50:7:1 and ~68:9:1, respectively. C:N ratios of *S. costatum* and *E. huxleyi* were 7:1 while this ratio was 8:1 in *P. micans*. When N and P were reduced, both C:N and C:P ratios increased. Cellular N:P ratio increased under P limitation and decreased under N limitation in *S. costatum* and *P. micans* (Table 2).

Cell sizes and total biovolumes

The average cell diameter and length of *S. costatum* were 4.0 ± 0.6 µm and 5.0 ± 0.9 µm, respectively. Large cell sizes (with diameters up to 8 µm and lengths up to 15 µm) were common for ammonium-grown *S. costatum*. The average cell width, length and height of *P. micans* were 31.0 ± 2.3 µm, 38.0 ± 2.9 µm and 15.0 ± 0.7 µm, respectively. In P- and ammonium-reduced flasks, cell height increased as much as width at the late stationary phase in this normally laterally flattened species. The average cell length, width and height of P limited cells were 32.0 ± 3.6 µm, 42.0 ± 2.8 µm, 26.0 ± 2.8 µm, respectively, on the 25th d of the experiment. This species divides laterally and this lateral enlargement may have occurred because of depressed division. Average cell diameter of *E. huxleyi* was 3.0 ± 0.9 µm and generally ranged from 2.0 to 4.0 µm.

Total cells of *P. micans* had the highest biovolume (74 mm^3 L^{-1}), followed by *S. costatum*, (~25 mm^3 L^{-1}) and *E. huxleyi* (~15 mm^3 L^{-1}) in F/2 medium at the end of the exponential phase (~400 × 10^6 cells L^{-1} and 62 $µm^3$; ~10 × 10^6 cells L^{-1} and 7401 $µm^3$; ~1,000 × 10^6 cells L^{-1} and 14 $µm^3$, for *S. costatum, P. micans* and *E. huxleyi,* respectively, see also "Materials and methods").

Chlorophyll *a* per cell and per cell volume, and carbon-to-chlorophyll *a* ratios

Average chl *a* per cell during the exponential phase (until cell numbers reach the highest concentration) was low under low ammonium treatments in *S. costatum* and *P. micans*. Average chl *a* per cell within F/2 medium during exponential phase was higher in *P. micans* (8 pg) than in *E. huxleyi* (0.2 pg) and *S. costatum* (0.4 pg), which was proportional to cell size. However, chl *a* content per cell volume was highest in *E. huxleyi* (~15 fg chl*a* μm^3), compared to *S. costatum* (~7 fg chl *a* μm^3) and *P. micans* (~1 fg chl *a* μm^3).

Chl *a* content per cell was highest in the early phases of exponential growth for *E. huxleyi* (~0.5 pg chl *a* $cell^{-1}$), *S. costatum* (~1 pg chl *a* $cell^{-1}$) and *P. micans* (~12 pg chl *a* $cell^{-1}$) under high nutrient conditions (Figs. 1a, 2a, 3b).

C:chl *a* ratios differed substantially from species to species (91 for *S. costatum*, 365 for *P. micans* and 50 for *E. huxleyi;* Table 2). When nutrient concentrations were reduced, C:chl *a* ratios were higher significantly (<0.05) at the end of the exponential phase. Unfortunately carbon concentrations were not measured during all growth phases, thus changes in this ratio during all growth phases (from the beginning of the experiment till the end), which might also vary significantly, are not shown.

Discussion

Bloom characteristics of phytoplankton in the sea are determined by complex relationships of several biological and physico-chemical factors. In laboratory studies, most of these factors are either eliminated or controlled, enabling evaluation of the influence of a specific parameter. In the present study, to observe effects of nutrients on phytoplankton, high nutrient concentrations, compared with many oceans and seas, were used to suppress possible effects of other laboratory conditions (such as light and CO_2 that might change slightly with differing cell density) on phytoplankton. Thus, although the laboratory conditions differ substantially from nature, laboratory studies provide important insights for understanding the functioning of biological systems, including effects of nutrients on bloom dynamics (total abundance, biovolume, chl *a* etc.) of phytoplankton. Chl *a*, being the most dominant photosynthetic pigment in phytoplankton, has a special importance since it is often used to estimate algal biomass (total biovolume or carbon) and production from *in situ* and remote sensing studies in nature. Most of these studies assume that there is a linear relationship between phytoplankton biomass and chl *a* concentration. However, there are several intrinsic factors affecting chl *a* contents of phytoplankton, as revealed once again in our study.

Both the total biovolume and chl *a* contents of all cells at a given nutrient concentration were found to differ to a large extent among species. Although the total biovolume (in F/2 medium) reached by *P. micans* cells (74 mm^3 L^{-1}) was 5 times higher than that of *E. huxleyi*, chl *a* concentration of *P. micans* was only as much as that of *E. huxleyi*. *Prorocentrum micans* had three times more total biovolume, but only half the chl *a* concentration of *S. costatum* (122 µg L^{-1}). *Emiliania huxleyi* chl *a* content per cell volume was higher than that of the other two species (Table 2). Similarly, Ediger et al. (accepted) reported that even if the total biovolume of *E. huxleyi* cells was low, it was still the major contributor to total chl *a* in the southwestern Black Sea in May 2001. This result shows that calculation of algal biovolume from chl *a* concentration alone may cause a high degree of error. Evaluation of algal biovolume, e. g. formed mainly by dinoflagellates (which have less chl *a* concentration compared to other phytoplankton groups), from satellites based on chl *a* will be an underestimate.

Chl *a* content per cell, too, differs among species as well as under different nutrient and light conditions. Chl *a* content per cell decreases under light saturated conditions (Cullen, 1982). Probably because of this, at a higher light intensity (55 W m^{-2} which equals ~250 µmol m^{-2} s^{-1}) Harrison et al. (1977) reported lower chl *a* per cell in *S. costatum* (0.14 pg) than in our study (0.43 pg at ~100 µmol m^{-2} s^{-1}) under high-nutrient conditions. Muggli and Harrison (1996) reported lower values of chl *a* content for *E. huxleyi* (0.11–0.13 pg $cell^{-1}$) compared to our results (0.3 pg $cell^{-1}$) under lower iron (100 nM), nitrate (30 µM) and phosphate (5 µM) concentrations. Muggli and Harrison (1997) noted much lower chl *a* in this species (0.049 pg chl *a* $cell^{-1}$) at much lower nutrient concentrations (10 µM NO_3^-, 1 µM $PO4^{-3}$ and 6 nM Fe^{+2}). Price et al. (1998) reported that coccolith bearing forms of *E. huxleyi* (C-cells) had higher chl *a* content (0.7 pg chl *a* $cell^{-1}$) compared to non-coccolith bearing cells (nonmotile, N-cells) (0.2 pg chl *a* $cell^{-1}$) under nutrient replete conditions. We also found a similar chl *a* content (0.2 pg chl *a* $cell^{-1}$) for our non-coccolith bearing cells of *E. huxleyi* (motile S-cells). Unfortunately, our chl *a* concentrations for *P. micans* could not be compared with other results due to the scarcity of related investigations on this species.

Additionally, we found that chl *a* content per cell changed during the growth phase of all three species: chl *a* per cell was highest in the early exponential growth under both high-nutrient and nutrient-limited conditions similar to other investigations with a different species (Young and Beardall, 2003a; Young and Beardall, 2003b).

Total POC concentrations of all three species cultures investigated (*E. huxleyi*, *S. costatum* and *P. micans*) were also different (9.7×10^3, 13.2×10^3 µg L^{-1} and 22.5×10^3 µg L^{-1}, respectively) under high-nutrient conditions and at the end of exponential phases. However, carbon contents of all three species were similar to values from the literature under similar conditions to our experiments.

The carbon content of *P. micans* (2100 pg cell^{-1}) found in the present study was close to that found by Menden-Deuer and Lessard (2000, 2735 pg cell^{-1}). *Skeletonema costatum* had a wide range of carbon content from about 8.5 pg to 125 pg (Strathmann, 1967; Harrison et al., 1977) and our value for this species was 40 pg cell^{-1}. The carbon content of *E. huxleyi* was 9.4 pg which was close to previously reported values (naked cells had 4.5–13 pg C cell^{-1}, and coccolith-bearing cells had 7.5–51 pg C cell^{-1}, Verity et al., 1992; Montagnes et al., 1994; Muggli and Harrison, 1996; Muggli and Harrison, 1997; Price et al., 1998).

Changes in nutrient concentrations often cause alterations in the C:chl *a* ratio of phytoplankton and, as a matter of fact, this ratio is used as an informative index of the nutritional state of field populations (Goldman, 1980). A strong correlation has been noted between the C:chl *a* ratio and nutrient (as well as light) limited relative growth rates of algae (Cullen, 1982; Cullen, 1992). Nutrient limitation promotes an increase in the C:chl *a* ratio (Harrison et al., 1977; Kiefer and Cullen, 1991; Landry et al., 2000) and C:chl *a* ratios were higher in the low-nutrient cultures in our study. However, this ratio also changes with different growth phases. Unfortunately, we have measured C content of cells only once, at the end of the exponential phase. The higher C:chl *a* ratio of *P. micans* compared to the other two species is in agreement with other published findings (Table 2). Under similar culture conditions, C:chl *a* ratio for dinoflagellates has been reported to be much higher than for diatoms (Chan, 1980). Steele and Baird (1962) reported that C:chl *a* ratio of *S. costatum* changed between 28 and 200 in the first 7 d of their experiment under nitrate limitation (170 µM) and similar light conditions to our study. The C:chl *a* ratio (90 at the 7th d of our experiment) for *S. costatum* at the end of the exponential phase in the present study was higher than the ratio (61) reported by Harrison et al. (1977) under high-nutrient conditions. According to Harrison et al. (1977), C:chl *a* ratio generally varies between 20 and 60 in healthy cells. However, many sources of variation in the C:chl *a* (e.g. due to changes in nutrients, light) can result in C ratios as low as 10 (Laws and Bannister, 1980) or as high as 250 or more (Holmes et al., 1967; Laws and Bannister, 1980; Redalje and Laws, 1981). We found that the measured C:chl *a* ratio increased by ~5 to 20 fold under nutrient limitation (phosphate, nitrate or ammonium) in all three species investigated in the present study. Changes in this ratio may be one of the major reasons resulting in differences in timing of total biovolume and chl *a* peaks, as was observed occasionally in field studies. For example, Ediger et al. (accepted) and Eker-Develi (2004) reported that chl *a* concentrations were high when total biovolume of phytoplankton was low or vice versa.

Similar to C:chl *a* ratio, *in vivo* fluorescence:chl *a* (*in vivo* Fl:chl *a*) ratio is also used for determining nutrient limitation (Cullen, 1982). The *in vivo* Fl:chl *a* ratio was highest under nutrient-limited conditions for all three species, suggesting a lower efficiency of electron transport (and hence of photosynthesis) for these species. Muggli and Harrison (1997) also reported this ratio to be higher under low iron concentration compared to high levels of this nutrient in *E. huxleyi*. The higher *in vivo* Fl:chl *a* ratio under limitation of phosphate compared to nitrate observed for *S. costatum* here (Fig. 4) may express that this species has a high phosphate requirement. Ammonium concentrations used in *P. micans* experiments apparently were either too high (thus probably toxic) or too low for the requirements of the phytoplankton species tested. Probably due to this reason, the *in vivo* Fl:chl *a* ratio was higher in cultures given ammonium than in nitrate- or phosphate-reduced cultures of all three species.

High P requirements of *S. costatum* were also evident from higher rate of P than of N consumption by this species. Inorganic N:P ratios in high-nutrient (F/2 medium) cultures of *S. costatum, P. micans* and *E. huxleyi* started at 24:1 and gradually increased to 3400:1, 120–170:1 and 24–29:1 during death phases of *S. costatum, P. micans* and *E. huxleyi* respectively (Figs. 1a, 2a, 3a). The low N:P ratio of *E. huxleyi* decreased further at the end of 3 months to 0.1, indicating severe N limitation in this species (N was almost entirely consumed whilst there was plenty of P, 7 µM). Organic N:P ratios in cells of both *S. costatum* and *P micans* were low (7–9:1) compared to the Redfield's ratio (16:1) which may also show higher P consumption under high-nutrient concentrations. Unfortunately, PP content of *E. huxleyi* was not measured. In addition, N preference of *E. huxleyi* was also evident from almost complete depletion of N on day 13 when N was reduced to 10 % of F/2 medium (indicating impossibility of further abundance increase, Fig. 3b) whilst about half of the P was still available in the P reduced samples (indicating possibility of further abundance increase, Fig. 3c). Similar to our findings, *E. huxleyi* has been often described as a good competitor at low P concentrations (Egg and Heimdal, 1994; Townsend et al., 1994; Vanbleijswijk et al., 1994; Maranon et al., 1996).

Probably due to inhibitory effects of high ammonium (880 µM), *S. costatum* could grow only after a prolonged adaptation period (~15 d) but not *P. micans*. Bates et al. (1993) reported that a similar concentration of ammonium was inhibitory to the diatom *Pseudo-nitzschia pungens f. multiseries*, more so than for *S. costatum*. In contrast to the present study, Bates et al. (1993) had previously acclimated their cultures to high ammonium concentrations.

In addition to determining limiting nutrient, molar C:N:P ratios in cells are important for calculating export production, nutrient based productivity, as well as in models of ocean productivity. In agreement with Geider and La Roche (2002)'s study, molar C:N ratios for all

three species in this study were close to Redfield ratio (6.6:1) under high-nutrient conditions and increased when nutrients were reduced (Table 2). Molar C:P ratios for *S. costatum* and *P. micans* were lower than Redfield's ratio (<106:1, Table 2) under high-nutrient conditions. However, when P was reduced this ratio increased about 5–7 times. Like in previously reported laboratory studies (Geider and La Roche, 2002), molar N:P ratio was lower (7–9:1) than Redfield ratio (i.e. 16:1) under high-nutrient conditions and this ratio decreased when N or ammonium was reduced or increased when P was reduced in both species (Table 2).

In this study, we found that generally growth rate of all three species did not decrease while total chl *a* concentrations declined in the late exponential phase when nutrient concentrations were reduced (Figs. 1a,b,c, Table 2a). It is often reported that when algal growth rate is reduced as a result of nutrient limitation, photosynthetic activity (as an indicator of chl *a*) is also reduced (Cullen, 1982; Beardall et al., 1991; Geider et al., 1993; Stolte et al., 2000; Litchman et al., 2003). However, in the present investigation growth rates did not change.

Our findings from laboratory experiments emphasize that chl *a* values in the natural habitat may not accurately indicate actual phytoplankton biomass. The different responses (abundance, *in vivo* fluorescence, C and chl *a* content, C:chl *a* ratio, nutrient uptake ratios) of each species to changing nutrient concentrations should be taken into account particularly in biomass, C or primary production studies based on chl *a* measurements from the field.

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