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In situ gross growth rates of *Emiliania huxleyi* in enclosures with different phosphate loadings revealed by diel changes in DNA content

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ABSTRACT: The *in situ* specific growth rate of the nanoplankton species *Emiliania huxleyi* can be successfully derived from analysis of diel DNA synthesis patterns. Calculated growth rates (μ_{DNA}) were in close agreement with growth rates determined from cell counts in laboratory cultures of *E. huxleyi*. For *E. huxleyi* populations in large outdoor enclosures (temp. = 7.9 to 10.2°C), μ_{DNA} ranged from 0.36 to 0.76 d^{-1}. Combining data on (net) changes in cell number of the enclosed populations with the calculated μ_{DNA} values provided information on the total specific loss rates of *E. huxleyi* (caused by grazing, viral infections and autolysis). The initial and mid-exponential phases of *E. huxleyi* blooming were characterized by relatively high μ_{DNA} values. At a later stage, following the depletion of nutrients in the water, μ_{DNA} decreased. Specific loss rates ranged from 0.07 to 0.63 d^{-1} and no particular trend in time was noticeable. *E. huxleyi* populations in enclosures with different phosphate loadings did not show significant differences in μ_{DNA}; in contrast, differences in loss rates were indisputable. Loss rates were low (0.07 to 0.35 d^{-1}) in fertilized enclosures with low and intermediate phosphate concentrations, allowing extensive *E. huxleyi* blooming. In the fertilized enclosure with high phosphate loadings (PO_4 > 2.6 mmol m^{-3}) and in the unfertilized oligotrophic enclosure losses were high (0.33 to 0.63 d^{-1}) preventing blooming of *E. huxleyi*. We concluded that nutrients were not limiting gross *E. huxleyi* growth but that they affected *E. huxleyi* losses by changing the phytoplankton composition and biomass.

KEY WORDS: Cell division · Diel cycle · DNA · *Emiliania huxleyi* · Flow cytometry · Growth rate · Loss rate · Phosphate · Phytoplankton · Prymnesiophyceae

INTRODUCTION

Emiliania huxleyi (Lohmann) Hay & Mohler is a coccolithophorid in the nanoplankton size class which occurs world-wide. Surface blooms of this characteristic species cover, on average, an area of 1.4×10^6 km^2 each year, of which 71% is located in the subpolar latitudes (Brown & Yoder 1994). Because *E. huxleyi* is a major pelagic producer of calcium carbonate and an important contributor to dimethyl sulphide emissions in the atmosphere, an understanding of *E. huxleyi* blooming contributes to the understanding of our global climate (Charlson et al. 1987, Holligan 1992, Malin et al. 1992, Westbroek et al. 1993).

During May 1992, we participated in a field study which had the aim of assessing the effects of nutrients (particularly phosphate) on *Emiliania huxleyi* blooming. Experiments were performed in large outdoor enclosures which varied in their nutrient loadings. Time series of phytoplankton abundance, biomass and production were described (Egge & Heimdal 1994, Van Bleijswijk et al. 1994a, Van der Wal et al. 1994). Abundance is a net parameter, affected by growth and loss processes. To understand the effects of nutrients on the abundance of *E. huxleyi* we needed to know the intrinsic growth rate of this specific species, independent of grazing, lysis and sedimentation. Our strategy was to determine the gross growth rate based on analysis of the *E. huxleyi* cell cycle.

Essentially, the rate at which cells in a (partially) phased population proceed through the 3 phases of the division cycle (G_1, S and G_2M phase, defined by Howard & Pelc 1953) was determined using DNA staining techniques and flow cytometry. The growth

rate was then calculated according to the mitotic index method of McDuff & Chisholm (1982) as improved by Carpenter & Chang (1988). This method has been tested with computer simulations (Carpenter & Chang 1988) and laboratory cultures of the dinoflagellate *Heterocapsa triquetra* (Chang & Carpenter 1988) and has already been applied in the field (Chang & Carpenter 1991, 1994, Vaulot et al. pers. comm.). It was shown that grazing does not affect the accuracy of the method (Chang & Dam 1993).

We describe here the first results of calculations of *in situ* specific growth rate for the coccolithophorid *Emiliania huxleyi* during 3 phases of bloom development: the onset of blooming, the exponential increase and the bloom peak. Special attention is given to the effect of nutrients (phosphate in particular) on gross *E. huxleyi* growth. Additionally, we present total *E. huxleyi* specific loss rates, which were determined by comparing gross and net growth rates.

MATERIALS AND METHODS

In order to test whether analysis of diel changes in DNA content would provide reliable estimates of the gross growth rate of *Emiliania huxleyi* populations, we first applied the method to laboratory cultures of this species. Three strains were involved: Ch24-90 (calcified type A, isolated in 1990 from the northern North Sea), Ch25-90 (calcified type B, isolated in 1990 from the northern North Sea) and LN (naked type A, clone of strain L which was isolated from the Oslo fjord in 1968 by Prof. Dr E. Paasche). Type A and B cells differ in their morphology and the immunological properties of their coccolith polysaccharide (Van Bleijswijk et al. 1991, Young & Westbroek 1991). Ch24-90 and Ch25-90 were grown at 15 and 10°C, respectively, in dilution cultures under various photon flux densities (ranging from 5.9 to 155.0 µmol photons m^{-2} s^{-1}) which were supplied in an artificial 16:8 h L:D cycle (Van Bleijswijk et al. 1994b). Of these cultures, 8 were light limited and 2 were phosphate limited. In addition, Ch24-90 and LN were sampled from batch cultures during temperature-limited growth (14°C).

Natural *Emiliania huxleyi* populations were sampled in 1992 from large seawater enclosures (volume 11 m^3) located in a fjord, 20 km south of the Norwegian city of Bergen (Egge & Heimdal 1994). The enclosures (and their duplicates), numbered 3, 4, 5 and 6, differed in their initial nutrient content. Enclosure 4 remained unfertilized and initially contained field concentrations of 5.0 mmol m^{-3} nitrate and 0.3 mmol m^{-3} phosphate. In Enclosures 3, 5 and 6, the initial concentration of nitrate was set at 18 mmol m^{-3} and of phosphate at 0.4, 4.5 and 1.0 mmol m^{-3}, respectively (low, high and intermediate phosphate loading). Duplicate enclosures showed a very similar development in their phytoplankton contents (Egge & Heimdal 1994). Moreover, the unfertilized enclosures and the surrounding fjord water behaved very similarly. It was concluded that the enclosure system itself had negligible effects on phytoplankton development and that the differences between enclosures largely resulted from the different nutrient regimes (see also Egge 1993).

In enclosures with low and intermediate phosphate loadings, intense blooms of *Emiliania huxleyi* developed, as determined from cell counts and measurements of the particulate inorganic carbon standing stock (Egge & Heimdal 1994, Van Bleijswijk et al. 1994a). High phosphate loadings favoured other phytoplankton species and had negative effects on the absolute abundance of *E. huxleyi*. *E. huxleyi* numbers also remained low in the unfertilized enclosures.

We took samples on 2–3 May, 11–12 May and 18–19 May (Enclosures 3 and 6 only). These dates corresponded with the initial phase, the mid-exponential growth phase and the peak of the *Emiliania huxleyi* bloom, respectively (Fig. 1). The natural L:D cycle was approximately 16:8 h. For further details on the 1992 enclosure experiments see Sarsia special volume 79 (1994).

For analysis of diel patterns in cellular DNA content, populations were sampled every second hour over a period of at least 24 h. Samples taken from the enclosures were first prefiltered over a 10 µm sieve to remove large organisms, aggregates and fecal pellets. Then, the <10 µm plankton fraction (which includes *Emiliania huxleyi*) was concentrated by filtering 1 to 3 l of the prefiltered subsamples under low pressure on polycarbonate filters (0.8 µm pore diameter, Isopore). Cells were washed off these filters by shaking the

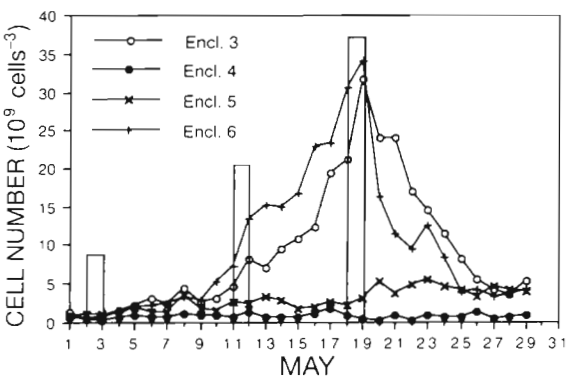

Fig. 1. *Emiliania huxleyi*. Time series of cell numbers in 4 natural seawater enclosures (data from J. K. Egge). Enclosures 3, 5 and 6 were fertilized with nitrate, and with phosphate in low, high and intermediate concentrations, respectively (see Table 1). Enclosure 4 was not fertilized. Bars indicate periods when gross growth rates were determined

filters in 5 ml of the corresponding prefiltered subsamples. The concentrated enclosure samples were then treated similarly to the laboratory culture samples. Cells were harvested in Eppendorf vessels by centrifugation (8 min at 450 × *g*), fixed in ice-cold methanol and stored at −50°C. Prior to analysis they were washed 2 times in a cold solution of phosphate-buffered saline (0.15 mol l^{-1} PBS, pH 7.9), and incubated in the same solution with RNAse (final concentrations: 40 mg l^{-1} RNAse A, Sigma R-4875) for 1 h at 10°C. Cellular DNA was stained with propidium iodide (PI) (final concentration: 5 mg l^{-1}) and analysed with a flow cytometer (EPICS CS, excitation wavelength 488 nm). Emission fluorescence of PI-stained nuclei was measured above 610 nm.

The stained preparations of the enclosure samples contained many plankton species from various trophic levels. Nevertheless, we were able to select *Emiliania huxleyi* populations in the flow cytometer histograms by presuming that their forward light scatter (angle: 0.5 to 19°) and side scatter (angle: 19 to 117°) characteristics were in close agreement with the corresponding signals of the unialgal laboratory cultures. Microscopical observations confirmed our interpretation of the flow cytometer histograms.

At least 15 000 *Emiliania huxleyi* cells were analysed per sample to obtain reliable DNA histograms. The G_1 and G_2M peaks were fitted by normal curves, the S phase by a polynomial curve using non-linear Marquardt algorithms ('Multicycle' software package, Phoenix Flow Systems). The fractions of cells in the different phases (f_{G_1}, f_S and f_{G_2M}) were then obtained from the area of the corresponding fitted curves.

In situ gross growth rates (μ_{DNA}, d^{-1}) were calculated according to the formula of Carpenter & Chang (1988) with minor revisions of Vaulot (1992) and given the restriction that $\mu_{DNA} < \mu_{max}$:

$$\mu_{DNA} = \frac{24}{(t_s)_\Sigma \cdot 2(t_2 - t_1 + k \cdot t_p)} \sum_{i=1}^{n} (t_s)_i \ \ln[1 + f_S(t_i) + f_{G_2M}(t_i)]$$

where μ_{max} = maximum possible growth rate (for *Emiliania huxleyi*: μ_{max} = 1.85 d^{-1}; Brand 1982); $(t_s)_\Sigma$ = total sample interval (h); t_2 = time (h) where $df_{G_2M}/dt = 0$; t_1 = time (h) where $df_S/dt = 0$; k = 0, 1, 2, 3, ...; t_p = entraining period (24 h for our samples); $(t_s)_i$ = sampling interval (h) of sample i; t_i = time (h) of obtaining the ith sample; f_S and f_{G_2M} = fraction of population in S and G_2M phase respectively. t_2 and t_1 were determined after fitting 4th degree polynomial functions through the data points of, respectively, f_{G_2M} and f_S against time.

Cell numbers were determined in fresh samples with a Fuchs-Rosenthal haemocytometer and after drying on cellulose acetate filters as described in Van Bleijswijk et al. (1994a). The net specific growth rate (μ_{cell}, d^{-1}) was averaged over 5 consecutive days and calculated as:

$$\mu_{cell} = \frac{\ln(N_d)_{i+1} - \ln(N_d)_i}{d_{i+1} - d_i}$$

where $(N_d)_{i+1}$ and $(N_d)_i$ are, respectively, the cell numbers at Day i+1 and Day i.

RESULTS AND DISCUSSION

During all diel sampling series, the DNA histograms clearly changed with time. This shows that phased cell division not only occurred in laboratory cultures, but also in the fertilized Enclosures 3, 5 and 6 as well as in the unmanipulated Enclosure 4. As an example, the results for Enclosure 3 on 18–19 May are presented in Fig. 2. At 12 and 14 h, 1 major peak is observed in the DNA histograms. This peak represents G_1 cells (gap 1 cells), which contain 1 DNA equivalent. At 17 h, the peak is broadened towards the right side because a part of the population (S cells) has started to synthesise extra DNA. Subsequently (20 to 26 h), the cluster of S cells gradually shifts to the right, as the mean cellular DNA content of the cluster increases due to the synthesis of DNA. It is noteworthy that the G_1 and S peaks move apart between 22 and 28 h. Apparently, no (or few) cells transgress the boundary between the G_1 and the S phase during this dark period. At 26 h a substantial fraction of the population contains 2 DNA equivalents (G_2M cells = gap 2 and mitosis cells). After 26 h the number of G_2M cells decreases whereas the number of G_1 cells increases due to mitosis which continues until 34 h (= 10 h in the preceding cycle).

The peaks in the DNA histograms showed a good fit by normal curves. The standard variabilities (CVs) of the fitted G_1 and G_2M peaks ranged from 6 to 15%. The ratio between the mean DNA contents of G_2M/G_1 cells ranged from 1.93 to 2.21.

Gross growth rates

In the laboratory cultures, μ_{cell} ranged from 0.20 to 1.24 d^{-1}. Over this entire range of growth rates μ_{cell} and μ_{DNA} were in close agreement (Fig. 3). The deviation of the regression line from the 1:1 relationship falls within the relative error of the method (18 to 22%) as calculated by Chang & Carpenter (1990) and Chang & Dam (1993). The result clearly shows that analysis of diel changes in cellular DNA content of *Emiliania huxleyi* provides reliable estimates of the gross growth rate. It is noteworthy that the success of the method does not depend on the growth limiting factor. This is because the length of the terminal phase ($S+G_2+M$) is determined for every population (Carpenter & Chang 1988).

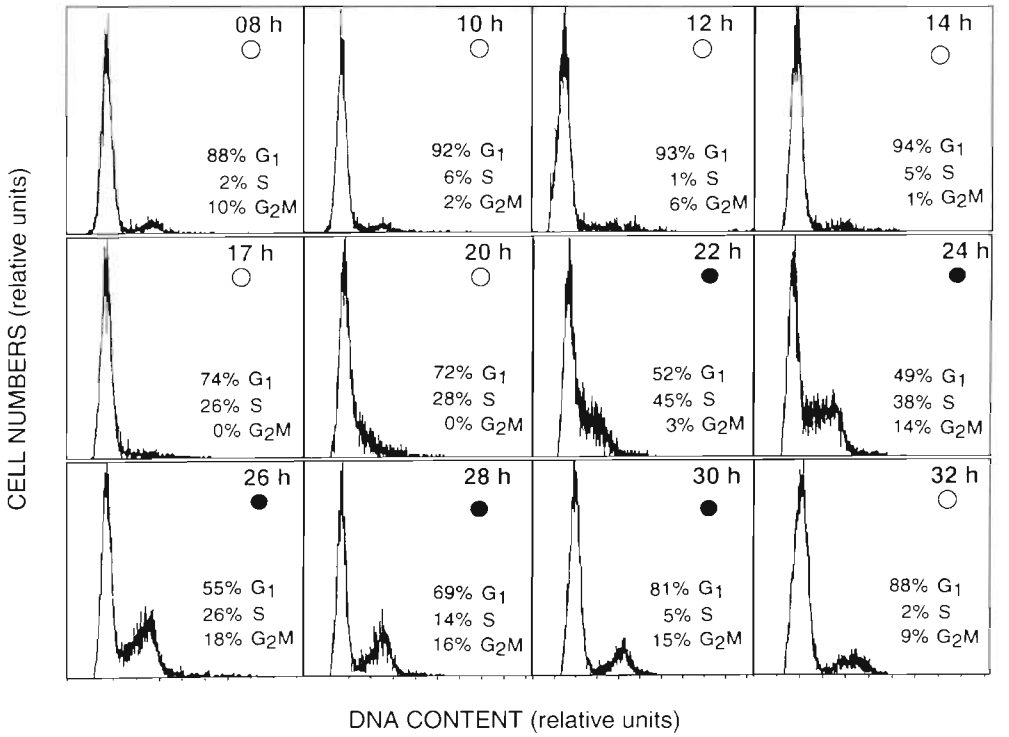

Fig. 2. *Emiliania huxleyi*. Diel changes in DNA content of the population in Enclosure 3 sampled on 18–19 May 1992. (○) Light period (06 to 22 h); (●) dark period (22 to 30 h). Percentages of the population in the G_1, S and G_2M phases are indicated in each panel

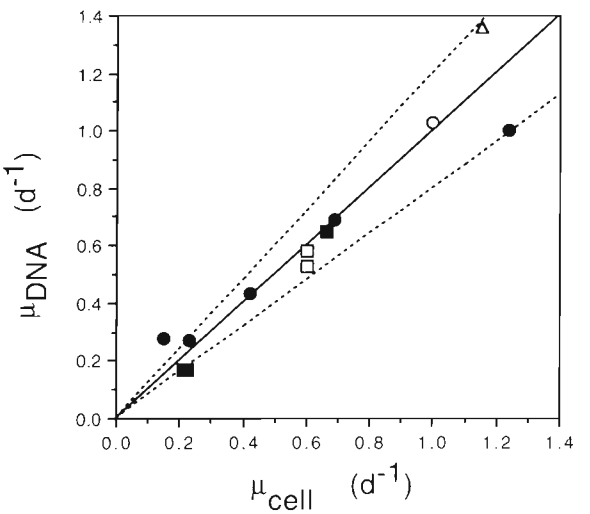

Fig. 3. *Emiliania huxleyi*. Calculated specific gross growth rate (μ_{DNA}, d^{-1}) versus the measured specific growth rate (μ_{cell}, d^{-1}) of laboratory cultures. μ_{DNA} was calculated on the basis of diel changes in cellular DNA content (Carpenter & Chang 1988); μ_{cell} was determined from changes in cell number. Continuous line = 1:1 relationship between μ_{DNA} and μ_{cell}. Dotted lines indicate interval in which datapoints do not significantly deviate from this 1:1 relationship assuming a relative error of 20% in μ_{DNA}. (●) Strain Ch24-90, light limited; (○) strain Ch24-90, temperature limited; (△) strain LN, temperature limited; (■) strain Ch25-90, light limited; (□) strain Ch25-90, phosphate limited

For the enclosure populations, calculated gross growth rates ranged from 0.36 to 0.76 d^{-1} (Table 1). At water temperatures of 7.9 to 8.2°C, growth rates of 0.63 to 0.76 d^{-1} are probably the maximum possible values. In well-adapted laboratory cultures of *Emiliania huxleyi*, grown at 10°C and under optimal light and nutrient conditions, growth rates of 0.81 d^{-1} have been measured (Van Bleijswijk et al. 1994b).

Gross growth rates (μ_{DNA}) were comparable on 2–3 May, during the initial phase of blooming, and on 11–12 May, during the mid-exponential growth phase, for all enclosures (Table 1). On 18–19 May, growth continued at the same rate (0.42 d^{-1}) in Enclosure 3 whereas in Enclosure 6, μ_{DNA} was reduced by 43% (to 0.36 d^{-1}). The reduction in μ_{DNA} in Enclosure 6 was preceded by a depletion of nutrients (N and P <0.1 mmol m^{-3} on 14, 15 and 16 May). After 17 May nutrient concentrations slightly increased, probably due to lysis and remineralization of phytoplankton cells.

Comparing *Emiliania huxleyi* populations in enclosures with different nutrient loadings, no significant differences were found in μ_{DNA} when relative errors of 20% were assumed. This indicates that the nutrient conditions did not limit gross *E. huxleyi* growth. Indirectly, this corroborates our hypothesis that *E. huxleyi* was growing at its maximum rate under the given temperature conditions.

Table 1 *Emiliania huxleyi*. Calculated gross specific growth rates (μ_{DNA}, d^{-1}) and measured net specific growth rates (μ_{cell}, d^{-1}) of semi-natural populations. Samples were taken from 4 outdoor enclosures with different nutrient conditions. μ_{DNA} was calculated on the basis of diel changes in cellular DNA content using the formula in the 'Materials and methods' with $k = 0$; μ_{cell} was determined from changes in *E. huxleyi* cell numbers on 5 consecutive days; *E. huxleyi* specific loss rate (d^{-1}) was calculated as loss = $\mu_{DNA} - \mu_{cell}$. PAR: photosynthetically available radiation (400 to 700 nm) at 2 m depth in the enclosures

Enclosure	Date (1992)	μ_{DNA} (d^{-1})	μ_{cell} (d^{-1})	Loss (d^{-1})	Cell no. (10^9 m^{-3})	Temp. (°C)	PAR (mol m^{-2} d^{-1})	$NH_4^+ + NO_x$ (mmol m^{-3})	PO_4^{3-} (mmol m^{-3})
3	2 May	0.51	0.44	0.07	0.52	7.9	10.1	14.34	0.25
4	2 May	0.64	0.13	0.51*	0.45	7.9	10.1	0.72	0.08
5	2 May	0.49	0.16	0.33*	1.00	7.9	12.0	13.95	4.69
6	2 May	0.59	0.39	0.20*	0.45	7.9	10.6	13.40	1.04
3	11 May	0.43	0.26	0.17*	4.58	8.2	8.6	10.84	0.08
4	11 May	0.63	0.00	0.63*	0.75	8.2	11.7	0.38	0.02
5	11 May	0.76	0.17	0.59*	2.70	8.2	6.5	2.73	2.66
6	11 May	0.63	0.28	0.35*	7.35	8.2	6.5	3.73	0.20
3	18 May	0.42	0.25	0.17*	21.20	10.2	8.9	5.94	0.26
6	18 May	0.36	0.11	0.25*	30.50	10.2	5.6	1.30	0.45

*Significant loss with 20% standard error in μ_{DNA}

Loss rates

We obtained estimates of the total *Emiliania huxleyi* specific loss rate (d^{-1}) in the enclosures by comparing the calculated gross growth rates with the averaged net growth rates determined from cell counts on 5 consecutive days. Loss rates ranged from 0.07 to 0.63 d^{-1} (Table 1) and no significant trend in time was noticeable. We do not know which factors are responsible for the calculated losses but it is clear that they determined the ultimate success of bloom formation. In general, our estimates of the total specific loss rates are higher than the grazing rates measured by several colleagues in Enclosures 3 and 6: combined data of Gonzales & Van der Wal (pers. comm.) and Nejstgaard et al. (1994) suggest a constant grazing on *E. huxleyi* by mesozooplankton throughout the experiment of 0.05 to 0.10 d^{-1} in Enclosures 3 and 6. In the same enclosures microzooplankton did not graze significantly on *E. huxleyi* (Nejstgaard et al. 1994). Viral-induced mortality could not be measured before 19 May (due to the methodology) but was very high afterwards in Enclosures 3 and 6 (1.3 d^{-1} in Enclosure 3 on 19–22 May and 2.0 d^{-1} in Enclosure 6 on 19–24 May; Egge & Heimdal 1994). General lysis was not quantified in any of the enclosures. The sedimentation rate was assumed to be low throughout the experiment due to the presence of an air lift.

Loss rates clearly differed in enclosures with different nutrient loadings (Table 1). Losses were 0.07 to 0.17 d^{-1} in Enclosure 3, 0.20 to 0.35 d^{-1} in Enclosure 6, 0.33 to 0.59 d^{-1} in Enclosure 5 and 0.51 to 0.63 d^{-1} in Enclosure 4. We had already observed that the relative contribution of *Emiliania huxleyi* to the total phytoplankton biomass decreased with increasing phosphate loading in the fertilized enclosures (Van Bleijswijk et al. 1994a). The present results show that at the same time the *E. huxleyi* specific loss rate increased with increasing P loading. For these P-related losses 3 causes can be considered: (1) increased impact of grazers on *E. huxleyi* due to changed phytoplankton composition; (2) higher activity of *E. huxleyi* specific viruses; (3) increased concentrations of toxins, produced by co-occurring phytoplankton species. The second possible mechanism for P-stimulated loss rates is based on the observations of Bratbak et al. (1993) that lysis of *E. huxleyi* was related to the activity of viruses and that the proliferation of these viruses was enhanced by high phosphate concentrations. With respect to the third cause we report that with increasing phosphate loadings the relative abundance of *Phaeocystis* sp. and dinoflagellates increased (Egge & Heimdal 1994). It is noteworthy that the phytoplankton standing stocks were rather high in the fertilized enclosures (0.3 and 0.6 g C m^{-3} on 2 and 11 May, respectively, in Enclosure 5) compared to natural *E. huxleyi* blooms where values of 0.04 to 0.3 g C m^{-3} are typical (Balch et al. 1991, Fernandez et al. 1993, Kristiansen et al. 1994); the artificial conditions may have enhanced inter-species interactions. An argument against the third possibility is that toxins would probably also affect the gross growth rate of the *E. huxleyi* populations, yet this was not observed.

In the unfertilized Enclosure 4 the high *Emiliania huxleyi* specific loss rates may be partly explained by a high impact of grazing. An equal quantity of grazers in all enclosures imposes a much larger relative effect when phytoplankton biomasses are low. Unfortunately

there are no experimental data available on meso- or microzooplankton grazing in this enclosure.

It is conceivable that the high losses in Enclosure 4 sustained the high gross growth rates observed in this enclosure at very low nutrient concentrations, and that the turnover rate of nutrients was high. If Enclosure 4 is indeed representative of oligotrophic conditions in the field, the production of calcium carbonate, and in general of refractive materials (rich in carbon) which are not readily recycled, may be much higher than the values which are presently accepted on the basis of cell counts or productivity measurements in blooms. The increasing amounts of particulate inorganic carbon (PIC; including attached and detached coccoliths) per *Emiliania huxleyi* cell in Enclosures 6, 3, 5 and 4 (6.7, 9.3, 11.8 and 17.7 ± 3.0 pg PIC $cell^{-1}$, respectively; Van Bleijswijk et al. 1994a) clearly illustrate the effect of high loss rates. In Enclosures 3 and 6 the PIC standing stock and the lipid biomarker concentrations increased at a rate comparable to the net *E. huxleyi* growth rate (Conte et al. 1994, Van Bleijswijk et al. 1994a). The increases in the PIC standing stock in Enclosures 5 and 4 were larger than the net increase in *E. huxleyi* cell numbers (resulting in the higher values of PIC per cell) but significantly smaller than the estimated gross growth rate. The observations indicate that with the loss of *E. huxleyi* cells, 75 to 80% of the corresponding amount of PIC is also assimilated.

CONCLUSIONS

At the onset of the *Emiliania huxleyi* blooms and during the mid-exponential increase of the populations, nutrient conditions did not limit *E. huxleyi* gross growth. Nutrient conditions did, however, influence *E. huxleyi* bloom formation: they clearly affected the specific loss rate of *E. huxleyi* populations, by profoundly changing the phytoplankton composition and biomass and thereby the impact of grazers on the coccolithophore. Under eutrophic conditions allopathy and phosphate-stimulated viral activity may also increase *E. huxleyi* losses.

In the future we hope to use the DNA method for mapping the horizontal and vertical variation of gross growth rate within *Emiliania huxleyi* blooms. Also, we want to see whether the absence of *E. huxleyi* blooms below 47° N in the Atlantic Ocean is due to slow gross growth. In general, we consider this method crucial for the verification in the field of hypotheses based on laboratory and model experiments, although it is quite laborious.

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