

Response of coccolithophorid *Emiliana huxleyi* to elevated partial pressure of CO₂ under nitrogen limitation

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ABSTRACT: Precipitation of calcium carbonate by phytoplankton in the photic oceanic layer is an important process regulating the carbon cycling and the exchange of CO₂ at the ocean-atmosphere interface. Previous experiments have demonstrated that, under nutrient-sufficient conditions, doubling the partial pressure of CO₂ (pCO₂) in seawater—a likely scenario for the end of the century—can significantly decrease both the rate of calcification by coccolithophorids and the ratio of inorganic to organic carbon production. The present work investigates the effects of high pCO₂ on calcification by the coccolithophore *Emiliana huxleyi* (Strain TW1) grown under nitrogen-limiting conditions, a situation that can also prevail in the ocean. Nitrogen limitation was achieved in NO₃-limited continuous cultures renewed at the rate of 0.5 d⁻¹ and exposed to a saturating light level. pCO₂ was increased from 400 to 700 ppm and controlled by bubbling CO₂-rich or CO₂-free air into the cultures. The pCO₂ shift has a rapid effect on cell physiology that occurs within 2 cell divisions subsequent to the perturbation. Net calcification rate (*C*) decreased by 25% and, in contrast to previous studies with N-replete cultures, gross community production (GCP) and dark community respiration (DCR) also decreased. These results suggest that increasing pCO₂ has no noticeable effect on the calcification/photosynthesis ratio (*C/P*) when cells of *E. huxleyi* are NO₃-limited.

KEY WORDS: Calcification · Carbon fixation · Coccolith · *Emiliana huxleyi* · Nitrate · Alkalinity · CO₂

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INTRODUCTION

The depression of marine calcification by elevated pCO₂ was first demonstrated in tropical coralline algae (Agegian 1985) and has subsequently been identified in other photosynthetic and calcifying organisms (reviewed by Gattuso et al. 1999a) and communities (Langdon et al. 2000, Leclercq et al. 2000, 2002). Although benthic calcification represents a significant component of the oceanic precipitation of calcium carbonate, the extent and significance of this response

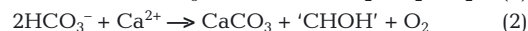
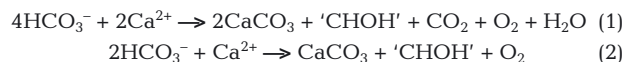
were unknown until recently, when it was shown that calcification of coccolithophorids, the major group of marine calcifying organisms, is also inhibited at elevated pCO₂ (Zondervan et al. 2001).

Calcification is a source of CO₂ to the water column (Ware et al. 1992, Frankignoulle et al. 1994). It therefore increases the sea-air CO₂ gradient and counteracts the uptake of CO₂ by the ocean. The biogeochemical significance of elevated pCO₂ on the future global carbon cycle remains uncertain for several reasons. Firstly, the magnitude of the decrease in calcification is highly vari-

able and depends upon whether organisms or communities are considered (Gattuso et al. 1999, Zondervan et al. 2001, Leclercq et al. 2002, Reynaud et al. in press). Some of this variability may result from an inadequate control of experimental conditions, particularly of the chemistry of the carbonate system. Second, the interactions on global scale between changes in pCO₂ and changes in other environmental parameters, such as temperature, have received very little attention and have the potential to explain some of the variability discussed above (Reynaud et al. in press). Third, most experiments have investigated acute responses but have not provided information on acclimation over short (daily) time scales. Fourth, the effect of this response on air–ocean CO₂ fluxes not only depends on its magnitude but also on other factors that are equally difficult to estimate, such as changes in seawater buffering capacity (Frankignoulle et al. 1994), the sedimentation rate of planktonic CaCO₃ particles (Buitenhuis et al. 2001), the effect of grazing by zooplankton (Harris 1994), and the response of the net photosynthetic uptake of CO₂ (Zondervan et al. 2001, Leclercq et al. 2002, Reynaud et al. in press). These uncertainties hamper the prediction of the effect of marine calcification on future air–ocean CO₂ fluxes, and, not surprisingly, the 2 attempts made so far have reached conflicting conclusions. Gattuso et al. (1999a) suggested that the release of CO₂ to the atmosphere due to calcification may not change significantly in the future, whereas Zondervan et al. (2001) suggested that it could increase significantly (see also Elderfield 2002).

Among all the substrates involved in primary production, dissolved inorganic carbon (DIC) is by far the most abundant (2 mmol C l⁻¹). For this reason, it was regarded for a long time as non-limiting for primary production (Lalli & Parsons 1994). By measuring the carbon specific growth rate of various phytoplanktonic species, including *Emiliana huxleyi*, at different DIC levels and constant pH, Clark & Flynn (2000) found that neither the rate nor the extent of primary productivity are significantly limited by the DIC concentration prevailing in the quasi-steady state conditions associated with oligotrophic areas. However, it is now well recognized that most marine species have developed enzymatic processes allowing them to accumulate intracellular DIC at levels that greatly exceed the external concentration (Raven & Johnston 1991). These carbon-concentrating mechanisms (CCM), which involve the active transport of bicarbonate and CO₂ through the cellular membrane, allow phytoplankton to use bicarbonate and CO₂ as sources of carbon for photosynthesis. Among the phytoplankton species, the calcifying strains of *E. huxleyi* have a particular relation with the DIC substrate because the production of calcium carbonate during calcification (C) is supposed to produce intracellular CO₂ that can be fixed during photosynthesis (P). According to the scheme

suggested by Nimer & Merrett (1995), calcification can produce extra CO₂ if its rate exceeds that of photosynthesis (Paasche 1964). Theoretically, the magnitude of CO₂ generation is thus a function of the C/P ratio, which can vary between 2 (Eq. 1) and 1 (Eq. 2) according to the following reactions (which do not take into account the buffering capacity of seawater):



Eqs. (1) and (2) remain hypothetical, since, as shown in numerous experimental studies, the C/P ratio can vary between 0 and more than 2 (Paasche 2002).

The aim of the present study was to build on the previous work of Riebesell et al. (2000) and Zondervan et al. (2001) by investigating the response of the coccolithophore *Emiliana huxleyi* to changes in pCO₂ under nitrogen-limited conditions. In contrast to these previous experiments, we chose to use continuous culture systems which, although technically more difficult to implement, allow the maintenance of phytoplankton in the required steady conditions of nutrient limitation. Continuous cultures also allow intensive and frequent sampling during prolonged time periods without significant variation in the volume, allowing accurate assessment of the acclimation properties of phytoplankton without adversely affecting the culture environment. Moreover, the possibility for growth and biomass to reach steady states greatly facilitates the control in real-time of pH and pCO₂ within narrow ranges. We ran 2 identical NO₃-limited chemostats of *E. huxleyi* in parallel. A dual control was available, as the cultures were initially maintained at the same pCO₂, with one of them subsequently subjected to the level of pCO₂ expected in the year 2100. In contrast to the classical pH regulation obtained by the addition of strong bases or acids, the seawater carbonate chemistry was manipulated by injecting various gas mixtures into the culture vessel. This experimental set-up was designed to provide information on 3 of the uncertainties mentioned above, specifically the physiological response under precise control of the culture conditions, the time response of acclimation observed over a prolonged time after perturbation, and the response of primary production. This experiment, carried out under nitrogen limitation, extends the knowledge of the pCO₂ effect on calcification to physiological conditions that also prevail in the ocean.

MATERIALS AND METHODS

Experimental design. The coccolithophore *Emiliana huxleyi* (Strain TW1) was provided by the Marine Biology and Biotechnology Laboratory of Caen University

(France). It was maintained in batch culture for 2 mo in conical flasks in 50 ml of K/2 medium (Keller et al. 1987). The flasks were placed in a growth chamber at a temperature of $17 \pm 0.1^\circ\text{C}$ under 14L:10D irradiance. Light was provided by fluorescent lamps giving a photon flux density (400 to 700 nm) of $170 \mu\text{mol photon m}^{-2} \text{s}^{-1}$.

We grew 2 chemostat cultures of the same strain under identical nutrient, temperature light and pCO₂ conditions until they had reached the same steady state; one culture was then subjected to an increase in pCO₂ (designated LH, i.e. for low to high pCO₂), while the other remained in unchanged conditions (designated LL, i.e. constant low pCO₂). This experimental set-up enabled comparison of the LH culture before and after the CO₂ shift as well as comparison with the control (LL) culture at the same date and time.

The 2 culture vessels consisted of water-jacketed 10 l cylinders (filled to 9.5 l) connected to a circulating water bath maintained at constant temperature ($17 \pm 0.5^\circ\text{C}$). The growth medium was prepared using 0.22 μm Millipore-filtered and autoclaved (105°C for 30 min) natural seawater and nutrient enrichments according to the K/2 formulation without ammonium. The medium was prepared in batches of 100 l (5 Nalgene tanks of 20 l connected together). After cooling and sterile addition of nutrients, the medium was transferred to the culture vessels through a 0.22 μm sterile filter (SpiralCap, Gelman). The nitrate concentration (NO₃), the limiting nutrient in fresh media, varied from 14.0 to 15.5 μM among batches. Phosphate concentration was 5 μM . The chemostat cultures were operated at a constant dilution rate (0.5 d^{-1}) that was periodically checked by weighing the incoming medium. The culture volume was large enough to minimize perturbation caused by intensive sampling. Chemostats were not refilled with fresh medium immediately after sampling, and the dilution rate was temporally increased by 6% 4 times per day, resulting in a daily averaged dilution rate increase of only 1.7%. Both cultures were subject to continuous gentle stirring and bubbling to insure homogeneity.

The cultures were grown under continuous light. Light was provided by an array of six 50 cm U-shaped, dimmable fluorescent tubes (OSRAM, DULUX®L, 2G11, 55W/12-950, LUMILUX DE LUXE, daylight) on each side of the set of 2 culture containers. The spectral characteristics of these tubes are given in Bruyant et al. (2001). The photosynthetic photon flux density was measured by immersing the spherical collector (QSL-100, Biospherical Instruments) in the center of the culture vessel. Irradiance was 570 and 580 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, in the LH and LL cultures, respectively.

Care was taken to avoid bacterial contamination of the cultures. However, axenicity could not be maintained, essentially because the pH electrodes had to be

removed from the chemostats each day for calibration. Nevertheless, the abundance of free and attached bacteria, counted daily by DAPI direct counts, remained similar to the cell abundance of *Emiliana huxleyi* ($<7 \times 10^8 \text{ cell l}^{-1}$), suggesting that bacterial carbon and activity were negligible compared to those of *E. huxleyi*. Indeed, no significant respiration was measured in culture filtrate (2 μm Nuclepore filter).

Nutrient and particle measurements. High-frequency monitoring of the chemostat cultures was performed throughout the experiment to obtain quasi real-time information on cell concentration and cell size distribution (hourly), and concentrations of nitrate (NO₃) and nitrite (NO₂) (twice a day). This information was essential to ensure that both cultures reached similar steady states before the pCO₂ shift since, at steady state, the growth rate was equal to the dilution rate. The concentrations of nitrate and nitrite were measured with a Technicon Auto-analyzer and an automated data-acquisition system (Malara & Sciandra 1991).

Cell concentration and size distribution were measured with an optical particle counter using the principle of light blockage (Hiac/Royco, Pacific Scientific Instruments). The instrument was calibrated with latex microspheres of 12 different sizes ranging from 1.7 to 250 μm . Comparative measurements of size and numbers made with the Coulter counter agreed reasonably with the Hiac counter for most of the phytoplankton species tested in our laboratory. Flow cytometer counts at different times during the experiments ($n = 53$) did not differ by more than 8% from simultaneous counts with the Hiac counter. This small difference could have been due to the presence of a small proportion of large free coccoliths which because of their scattering properties may have been counted as coccolithophorids. Another source of variation is the presence of dead cells. Since the Hiac counter counts all particles which have a refractive index significantly different from that of seawater, it cannot differentiate between dead and live particles. Because of their external coccosphere, dead empty coccolithophorids can retain their original shape longer than other phytoplanktonic cells, and thus their contribution to the entire population can bias cell counts. Nevertheless, these sources of error should not be significant, since the Hiac counts were generally slightly lower than the flow cytometer counts.

Prior to counting and sizing of *Emiliana huxleyi*, the cultures were diluted with an automated, computer-controlled system consisting of peristaltic pumps, solenoid valves, and a syringe (Bernard et al. 1996). The coefficient of variation for these replicates was less than 3%, and the data were subsequently averaged. The mean cell volume (V) was calculated for the 1.6 to 7.0 μm size classes, the lower detection limit of the Hiac sensor and the upper size of *E. huxleyi*, respectively.

Alkalinity and pCO₂ control. Triplicate 20 ml samples were collected every day at 8:00 h in both chemostats as well as in the culture medium for determination of total alkalinity (TA). They were immediately filtered onto glass-fiber filters (Whatman GF/C) and analyzed. TA was determined potentiometrically using the Gran method with a glass combination electrode (Orion 8102SC) calibrated on the National Bureau of Standards (US). pH scale and 0.10 N hydrochloric acid (Merck 109060). The precision of the measurements was assessed against a standard provided by A. G. Dickson (University of California, San Diego). The average of 8 replicate titrations was 2.9 $\mu\text{mol kg}^{-1}$ higher than the nominal TA of the standard (2189.56 ± 0.004 vs 2186.62 ± 0.36 mol kg^{-1} ; mean \pm 95 % confidence limits).

The pCO₂ in the chemostats was controlled using a pH-stat approach: pH was regulated by adjusting the delivery of 3 gases (air, pure CO₂ and CO₂-free air obtained by passing air through a scrubber filled with soda lime) into the cultures using solenoid valves. Because TA varied in the cultures, depending on the magnitude of the rates of calcification, the desired pH was determined daily. It was calculated using TA and the desired pCO₂ as described by Zeebe & Wolf-Gladrow (2001). The pH was measured at 1 s intervals using glass combination electrodes (Orion 8102SC) calibrated daily using the Seawater scale buffers Tris and 2-aminopyridine (Dickson 1993). It varied from 7.82 in the LH culture after the pCO₂ increase to 8.02 in both cultures before time zero (t_0) (Table 1). Temperature was also measured at 1 s intervals with Pt100 platinum resistance sensors.

Table 1. *Emiliana huxleyi*. Mean (SD) values of the dissolved inorganic carbon system components for the 2 cultures LH (low to high pCO₂) and LL (continuously low pCO₂), before and after shift in partial pressure of CO₂ (pCO₂) in the LH culture at t_0 (time zero). Var: percentage variation between and after t_0

Culture	pH	pCO ₂ (ppm)	HCO ₃ ⁻ ($\mu\text{M kg}^{-1}$)	CO ₃ ²⁻ ($\mu\text{M kg}^{-1}$)	CO ₂ ($\mu\text{M kg}^{-1}$)
Before t_0					
LH	8.021 (0.009)	412 (12)	1814 (20)	162 (4)	15.1 (0.5)
LL	8.014 (0.011)	417 (12)	1805 (8)	159 (4)	15.3 (0.4)
After t_0					
LH	7.824 (0.006)	708 (13)	1976 (24)	112 (2)	25.9 (0.5)
LL	8.022 (0.033)	404 (41)	1769 (25)	158 (10)	14.8 (1.5)
Var					
LH	-2.45	72	8.9	-31	72
LL	0.10	-3.1	-1.9	-0.3	-2.9

When the measured pH was lower or higher than that desired, CO₂-free air or a mixture of air and pure CO₂ was bubbled into the chemostats to raise or lower the pH, respectively. All calculations related to the seawater carbonate system were made according to Dickson & Goyet (1994). The accuracy of the pCO₂ control was ± 12 to ± 41 μat (1 SD; Table 1). pCO₂ increased by 72 % in the LH culture, with no overlap of the standard deviations. A CO₂ control was run for both chemostats during most of Phase 1 of the experiment. An electrical interference scrambled the pH signals in both cultures during the early part of Phase 2 and could not be suppressed at the time. The pCO₂ control system of the LH culture was therefore left in operation while ambient air was bubbled through the LL culture, whose control system was switched off. The pH in the LL culture was then measured several times a day by shifting the pH electrode from the LH to the LL culture. This technical problem did not affect the experiment, as pCO₂ values in the LL culture before and after t_0 were similar (Fig. 1), with overlapping SD.

Particulate organic and inorganic carbon and nitrogen. The concentrations of particulate carbon (PC), and nitrogen (PN), were determined every day or twice a day on triplicate samples. We filtered 50 ml onto precombusted glass-fiber filters (Whatman GF/F); these were then dried at 60°C and analyzed with a LECO 900 CHN analyzer. Particulate organic carbon (POC) was measured with the CHN analyzer using acidified samples (1 h above HCl fumes). The replicates typically agreed to within 5%. Particulate inorganic carbon (PIC) contained in free and attached coccoliths was calculated by the difference between PC and POC.

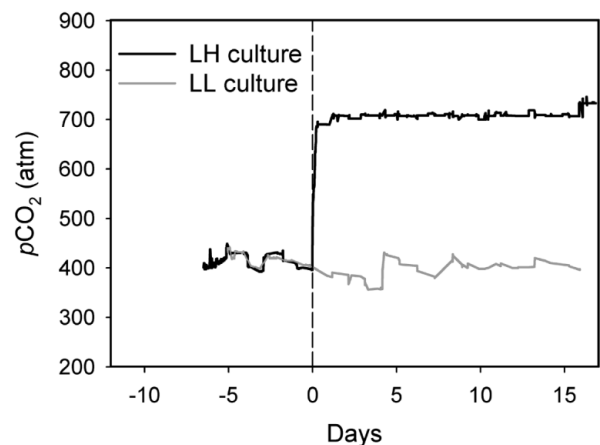


Fig. 1. Control of partial pressure of CO₂ (pCO₂) in LH and LL (low to high pCO₂) and LL (continuously low pCO₂) chemostats. At time zero (t_0) pCO₂ was increased in LH culture and maintained constant around 707 ppm, but was left unchanged in LL culture

Calcification and carbon fixation. Net calcification was measured by 2 independent techniques: budgeting the change in TA and the change of PIC in the chemostats. The evolution of TA in continuous cultures results from physical exchanges and biological processes. Addition of the enriched medium to the chemostat and its subsequent removal are termed positive and negative exchange, respectively. Precipitation and dissolution of CaCO₃ are termed sink and source, respectively, with a $\Delta\text{CaCO}_3:\Delta\text{TA}$ ratio of -1 mol: 2 equiv. An uptake of 1 mol nitrate and 1 mol phosphate increases TA by 2 mol. The change in TA due to nutrient uptake was negligible compared to that for calcification and CaCO₃ dissolution (Brewer & Goldman 1976). The significant fluxes in TA can thus be combined by the following equation to derive the instantaneous resulting alkalinity change:

$$\frac{d\text{TA}}{dt} = D(\text{TA}_i - \text{TA}) - Cn \quad (3)$$

where D (d⁻¹) and TA_i ($\mu\text{equiv. l}^{-1}$) are the dilution rate (ratio of flow rate to volume of the culture) and the TA of the culture medium, respectively. (TA_i varied from 2537 to 2607 $\mu\text{equiv. l}^{-1}$); TA and n (cell l^{-1}) are TA in the chemostat and the algal concentration, respectively; and C is the net cell calcification rate ($\text{pg C cell}^{-1} \text{d}^{-1}$). Rearranging Eq. (3), the mean cell calcification rate can be calculated as:

$$C = \frac{1}{n} \left(D(\text{TA}_i - \text{TA}) - \frac{d\text{TA}}{dt} \right) \quad (4)$$

Eq. (4) can be approximated by its discretized expression calculated for a significant time interval, Δt :

$$\bar{C} = \frac{1}{n(0)} \left(D(\text{TA}_i - \text{TA}(0)) - \frac{\Delta\text{TA}}{\Delta t} \right) \quad (5)$$

where $\text{TA}(0)$ and $n(0)$ are the values of TA and n at the beginning of the time interval Δt , respectively; ΔTA and \bar{C} are the difference in TA and the average rate of net cellular calcification calculated during this time interval, respectively; TA was measured daily ($\Delta t = 1$ d) in the renewal medium and in the 2 cultures. \bar{C} can be considered as a good approximation of C if the system is linear during Δt , i.e. if

$$\frac{\Delta\text{TA}}{\Delta t} \approx \frac{d\text{TA}}{dt} \quad \text{and if } n(t) \approx n(0)$$

and if the dilution rate D and the TA in the enrichment medium, (TA_i), remain stable during Δt .

The PIC concentration in the chemostats increased and decreased respectively in response to net calcification and dilution:

$$\frac{d\text{PIC}}{dt} = Cn - D\text{PIC} \quad (6)$$

At steady state, the values of PIC and n are thus sufficient to determine the cellular calcification rate C :

$$C = D \frac{\text{PIC}}{n} \quad (7)$$

PIC is the carbon concentration of attached and detached coccoliths. To obtain an unbiased estimation of C from Eq. (7), it is necessary that free coccoliths are efficiently retained, which was the case with the GF/F filters.

Similarly, the net cellular synthesis of POC in the chemostats, P ($\text{pg C cell}^{-1} \text{d}^{-1}$), can be calculated at steady state as:

$$P = D \frac{\text{POC}}{n} \quad (8)$$

Gross (GCP) and net community production (NCP) and dark community respiration (DCR). Cultures were siphoned from chemostat vessels into 12 borosilicate glass bottles through silicon tubing, overflowing each sample bottle by twice its volume. Four 25 ml dissolved oxygen sample bottles were fixed immediately with Winkler reagents for measurement of O₂ concentrations at t_0 ; 4 dissolved oxygen sample bottles were incubated in the light, and a further 4 bottles were incubated in the dark. Samples were fixed after either 4 or 11 h of incubation and stored under water pending analysis (within 24 h). Dissolved oxygen concentration was measured with an automated whole-bottle Winkler titration system using a photometric endpoint (Williams & Jenkinson 1982). NCP was calculated as the 24 h change in dissolved oxygen in the light bottle; DCR was calculated as the 24 h change of dissolved oxygen in the dark bottles; GCP was calculated as the sum of NCP and DCR. All rates are reported in $\text{pmol O}_2 \text{cell}^{-1} \text{d}^{-1}$.

RESULTS

Chemostat culture of *Emiliana huxleyi*

During the initial phase of the experiment, cells grew exponentially at a mean rate of 1.07 and 1.10 d⁻¹ in the LH and LL cultures (Fig. 2a), respectively. The continuous mode began in the chemostats with a dilution rate of 0.5 d⁻¹ when NO₃ became exhausted. Cell density reached identical steady states (around $4 \times 10^8 \text{ cell l}^{-1}$) in the 2 cultures 10 d before the shift of pCO₂ in the LH chemostat. At the end of the experiment (Day 16), the supply of fresh medium was interrupted and the cultures were grown in batch mode. This induced an immediate and similar increase in the cell density in both chemostats.

The increase of pCO₂ in the LH culture had no noticeable effect on the cell concentration compared to

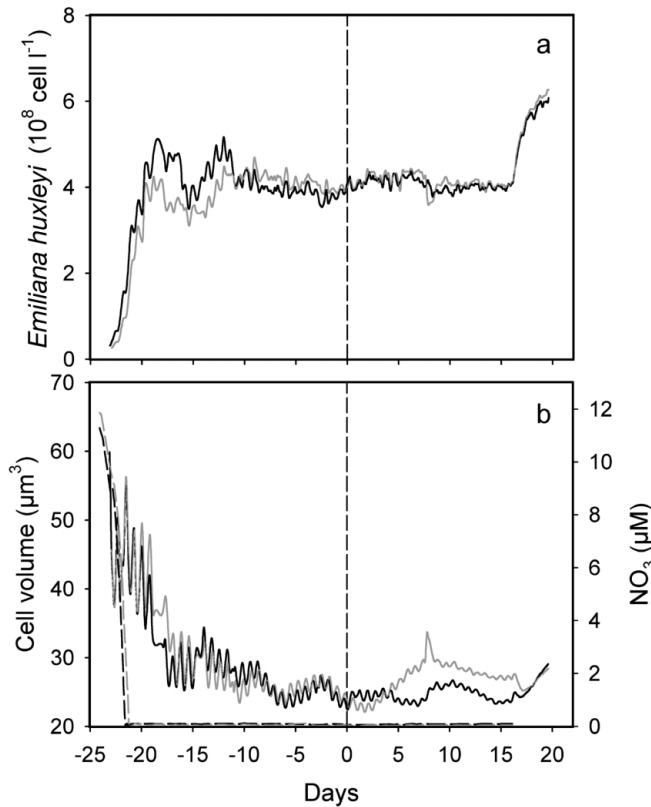


Fig. 2. *Emiliana huxleyi*. Time change in (a) cell concentration, and (b) mean cell volume (continuous lines) and NO_3 concentration (dashed lines) in LL (grey lines) and LH (black lines) chemostats before and after pCO_2 shift in LH culture at Day 0

that of the control LL culture. During the steady state observed before and after the pCO_2 shift, the mean generation time was 1.39 d in both chemostats (i.e.

$\ln(2)/0.5$). Cell density and mean cell volume (Fig. 2b) exhibited significant short-term oscillations throughout the experiment, suggesting that the algal population remained partly synchronized despite the continuous-light conditions of the 2 chemostats.

Superimposed on these short-term oscillations was a decreasing trend in the cell volume observed after NO_3 exhaustion in the 2 cultures (Fig. 2b). This agrees with the findings of Riegman et al. (2000), who reported a strong decrease in cell volume under nitrate limitation. The populations displayed identical evolutions of cell volume prior to the pCO_2 shift, but the trends subsequently diverged, with significantly lower cell volumes in the LH culture. This difference disappeared as soon as regulation of pCO_2 and dilution were voluntarily stopped at Day 16.

Net primary production and respiration

Total particulate nitrogen in the 2 chemostats was stable throughout the experiment, but exceeded the nitrate concentration in the renewal medium by 10%. This difference could be due to constant errors in the dosage of particulate organic nitrogen (PON) and DIN or to other sources of nitrogen present in small quantities in the natural seawater used for medium preparation. Some clones of *Emiliana huxleyi* have been shown to be able to use other nitrogen compounds such as amino acids (Ietswaart et al. 1994) and urea (Palenik & Henson 1997). However, given the small difference between PON and DIN and given the high C/N ratio measured (Table 2), one can reasonably consider that cells were effectively nitrogen-limited.

Table 2. *Emiliana huxleyi*. Mean (SD) pCO_2 , cell concentration (n), mean cell volume (V), organic (C_{POC}) and inorganic (C_{PIC}) cell carbon, cell nitrogen (C_{PON}), particulate organic carbon to particulate nitrogen ratio (C/N), and calcification to photosynthesis ratio (C/P) before and after pCO_2 shift in LH and LL cultures. Mean values before and after time 0 (t_0) were calculated from all measurements before t_0 , and from last 8 measurements after t_0 , respectively, all of which were representative of a steady state (see Fig. 3). Var: relative variation before and after t_0

Culture	pCO_2 (ppm)	n (10^8 l^{-1})	V (μm^3)	C_{POC} (pg cell $^{-1}$)	C_{PIC} (pg cell $^{-1}$)	C_{PON} (pg cell $^{-1}$)	C/N (at.)	C/P
Before t_0								
LH	412 (13)	3.89 (0.09)	24.64 (1.21)	5.09 (0.15)	4.45 (0.27)	0.57 (0.04)	10.50 (0.61)	0.87 (0.05)
LL	417 (12)	4.05 (0.08)	25.58 (1.20)	5.27 (0.10)	4.60 (0.15)	0.59 (0.07)	10.48 (1.06)	0.87 (0.04)
After t_0								
LH	708 (13)	4.03 (0.18)	24.92 (0.92)	4.37 (0.23)	3.92 (0.36)	0.55 (0.01)	9.25 (0.55)	0.90 (0.11)
LL	404 (41)	4.10 (0.14)	28.13 (0.94)	5.34 (0.32)	4.71 (0.24)	0.54 (0.03)	11.52 (0.86)	0.89 (0.09)
Var								
LH	0.72	0.04	0.01	-0.14	-0.12	-0.03	-0.12	0.03
LL	-0.03	0.01	0.10	0.01	0.02	-0.08	0.10	0.01

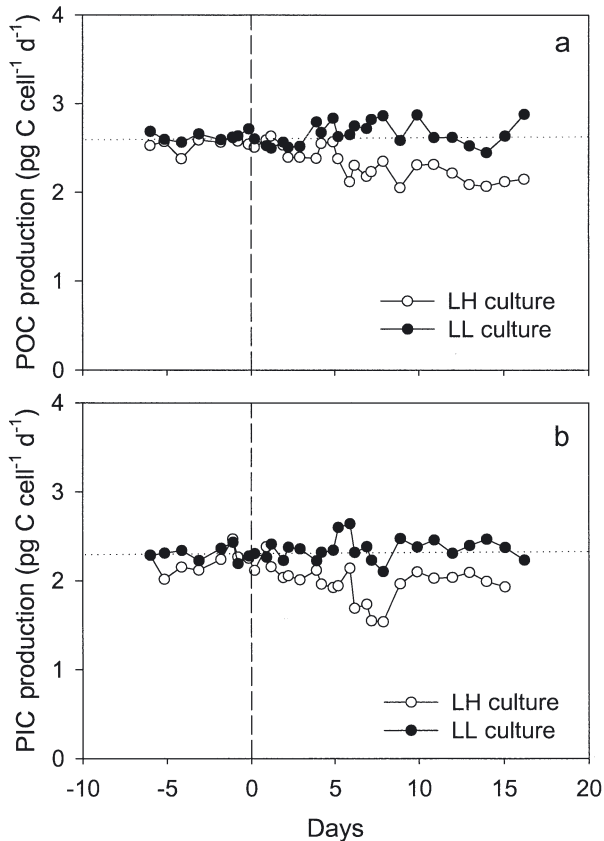


Fig. 3. *Emiliana huxleyi*. Net cellular production of (a) organic and (b) inorganic carbon in LH and LL cultures, before and after pCO₂ shift in LH culture at Day 0

The mean cell content of organic carbon measured in this experiment (Table 2) agrees with other data for *Emiliana huxleyi* (Paasche 1998, 1999). Production of organic carbon P remained stable in the control LL culture throughout the experiment, and was very close to the values in the LH culture before the pCO₂ increase (Fig. 3a & Table 3). In contrast, a significant decrease in particulate carbon production occurred consecutively with the pCO₂ increase in the LH culture. Since the cell abundances were very similar and constant in both the LH and LL cultures, the changes in organic and inorganic carbon production expressed as unit of culture volume (data not shown) exhibited a pattern similar to those expressed as cell unit.

GCP remained at around 0.26 ± 0.02 pmol O₂ cell⁻¹ d⁻¹ in both chemostats (Table 3). A decrease of 8.4% was observed after the pCO₂ shift in the LH chemostat. A decrease of 24% was observed for DCR, with a mean specific rate of 0.08 ± 0.02 pmol O₂ cell⁻¹ d⁻¹. Consequently, NCP increased by 16.4%, with a mean specific rate of 0.18 ± 0.02 pmol O₂ cell⁻¹ d⁻¹. The NCP fluxes after the pCO₂ shift in the 2 chemostats were significantly

different, essentially due to the decrease in DCR fluxes in the LH chemostat. The average ratio of GCP/DCR, an index of the net efficiency of the cells to fix carbon into the organic pool, was 3.1 ± 0.7 , which is higher than previously reported for batch cultures (1.6: Grande et al. 1989, and 2.6: Eppley & Sloan 1965). This ratio indicates that nearly 68% of the inorganic carbon fixed by the cell through photosynthesis was converted into organic biomass, the remaining 32% being released into the inorganic carbon pool. The apparent PQ value, which is the molar ratio of NCP(O₂) to NCP(C) determined by the POC variation, is 0.83 ± 0.11 . This apparent PQ value is low compared to the value of 1.2 to 1.4 reported under NO₃ supply (Eppley & Sloan 1965, Laws 1991). A limitation of the production rate GCP(O₂) by 30% due to nitrogen limitation during the batch-incubation could explain the low apparent PQ.

TA and calcification rate

TA was very stable in the enrichment medium, except in the last batch used from Day 5 onwards

Table 3. *Emiliana huxleyi*. Mean (SD) calcification (C), production of particulate organic carbon (P), (pgC cell⁻¹ d⁻¹) gross community production (GCP), net community production (NCP) and dark community production (DCR) rates (pmol O₂ cell⁻¹ d⁻¹) before and after t_0 in the LH and LL cultures. Student's t -tests indicate probability that values before and after t_0 or values in LH and LL cultures are equal (H_0)

Parameter	Before t_0	After t_0	t -test
C (Eq. 5)			
LH	2.31 (0.25)	1.76 (0.12)	$p < 0.0002$
LL	2.35 (0.19)	2.38 (0.08)	$p > 0.17$
t -test	$p > 0.7$	$p < 0.0001$	
C (Eq. 7)			
LH	2.22 (0.13)	2.02 (0.06)	$p < 0.002$
LL	2.30 (0.08)	2.38 (0.08)	$p > 0.03$
t -test	$p > 0.08$	$p < 0.0001$	
P (Eq. 8)			
LH	2.55 (0.07)	2.17 (0.10)	$p < 0.0001$
LL	2.63 (0.05)	2.65 (0.15)	$p > 0.4$
t -test	$p > 0.03$	$p < 0.0001$	
GCP			
LH	0.28 (0.00)	0.25 (0.02)	$p > 0.2$
LL	0.26 (0.02)	0.26 (0.02)	$p > 0.7$
t -test	$p > 0.2$	$p > 0.2$	
NCP			
LH	0.16 (0.05)	0.18 (0.02)	$p > 0.3$
LL	0.17 (0.05)	0.19 (0.01)	$p > 0.5$
t -test	$p > 0.8$	$p < 0.02$	
DCR			
LH	0.10 (0.04)	0.08 (0.01)	$p > 0.05$
LL	0.09 (0.03)	0.07 (0.01)	$p > 0.1$
t -test	$p > 0.7$	$p > 0.3$	

(Fig. 4a). The slightly lower TA measured in this batch can be explained by the change of seawater supply used for its preparation (the stock of seawater used to prepare the previous batches had been exhausted). The marked difference in TA levels between the enrichment medium and the 2 cultures indicates net calcification. Prior to t_0 , a very small difference in TA could be observed between the 2 cultures. This had completely disappeared by the time of the $p\text{CO}_2$ shift, suggesting that growth conditions were very similar for the 2 populations of *Emiliana huxleyi* until that time. Following the $p\text{CO}_2$ change, the TA in Chemostat LH increased significantly compared to the control culture LL.

We used the chemostat property that any dosed quantities can be converted into fluxes to estimate the calcification rate, by budgeting alkalinity in the renewal medium and in the cultures day by day (Eq. 5). Interestingly, this simple procedure allows the estimation of cellular calcification without manipulating cells, excepted for counting. Some noise around the average

tendencies (Fig. 4b) could have been due to the relatively low levels of cell calcification (1.5 to 3.2 $\mu\text{g C cell}^{-1} \text{d}^{-1}$), to the variations in effective dilution rate resulting from intensive sampling, and/or to the replacement of batches of medium. Nevertheless, it is obvious that the effects of doubling $p\text{CO}_2$ on calcite precipitation took place 2 d (i.e. 1 or 2 generation times) after the $p\text{CO}_2$ shift, and stabilized 7 to 8 d thereafter.

The decrease of calcification (C) in the LH culture was significant not only compared to the rate measured before the shift in the same chemostat, but also compared to the rate measured in the control culture LL (Table 3). In contrast, there was no significant difference in C before and after t_0 in the control culture LL. Assuming that the last 8 data points for LH and LL in Fig. 4b are representative of a steady-state activity, the increase in $p\text{CO}_2$ induced a 25% decrease in the rate of net calcification. The average rates of net calcification calculated from PIC measurements (see Eq. 7) agree qualitatively with the rates estimated from TA budget (Eq. 5), but the decrease in C estimated by the PIC measurement method (Eq. 7) was only 11% in the perturbed LH culture.

DISCUSSION

Since the pioneer work of Paasche (1964), the effects of DIC composition on calcification and growth have been studied in exponential or stationary batch cultures. Our experiment is the first to investigate the effect of an increase of $p\text{CO}_2$ on the calcification of N-limited cells of *Emiliana huxleyi* in continuous cultures. Our approach also differs from the previous studies in that the $p\text{CO}_2$ was not regulated by adding base or acid to the cultures, but by bubbling CO_2 -enriched or free air into the culture to regulate the pH at values calculated from measured alkalinity. Finally, measurements acquired in a long-term chemostat experiment reinforced the significance of the differences observed between high and low $p\text{CO}_2$ conditions.

One of the most surprising observations was the presence of persistent cell-size oscillations in cultures maintained in steady conditions of light, temperature and nutrient supply. Such a phenomenon has occasionally been observed using our culturing system in continuous cultures of *Thalassiosira pseudonana* and *Dunaliella tertiolecta* grown under similar conditions. It is remarkable that these oscillations (visible by means of the automated system of particle acquisition) continued throughout the entire experiment, except when dilution was interrupted on Day 16. Although slightly less obvious, oscillations in cell number were

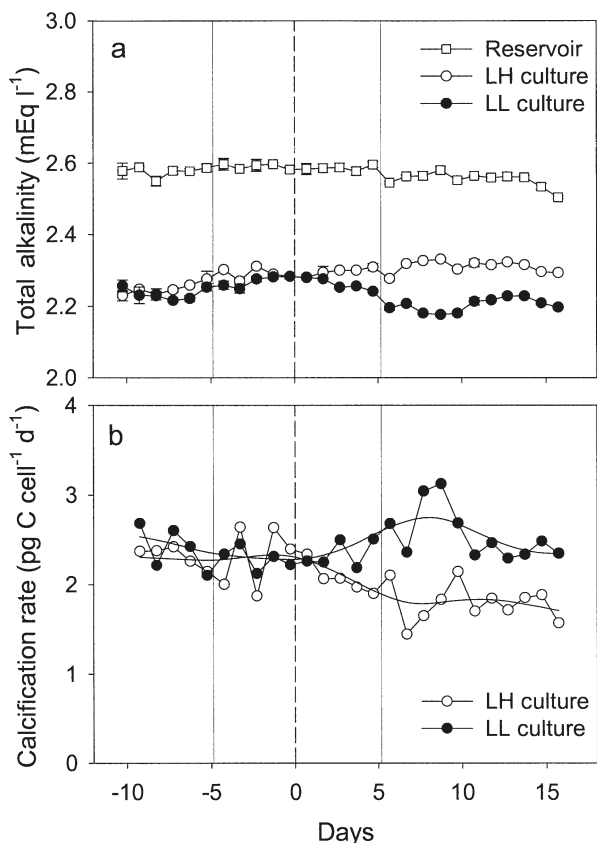


Fig. 4. *Emiliana huxleyi*. Time variation in (a) total alkalinity (TA) in renewal medium (Reservoir) and in LH and LL chemostats, and (b) calcification rate measured from Eq. (5). Solid vertical lines indicate the times when medium batches were changed

also observed. When examined in detail, oscillations of size and concentration were out of phase, suggesting that they resulted from synchronization of cell division. Fourier analysis revealed that the predominant periods were similar in the 2 cultures: 14.6 and 15.1 h for the LH and LL cultures, respectively, and were very close to the light period of the 14L:10D cycle used for the batch cultures that served as inoculum for the chemostats. It is also interesting that the characteristic period of 15 h was almost half the generation time (33 h) of *Emiliana huxleyi* in this experiment. Synchronization of cell population under constant growth-nutrient limitation continuous cultures has been suggested by theoretical investigations (Pascual & Caswell 1997), and was explained by the fact that nutrient assimilation and division are consecutive processes in the cell cycle, the latter process taking place after the completion of the former (Vaulot et al. 1987).

The effects of nitrate on growth and calcification of *Emiliana huxleyi* have been investigated in several studies. Nimer & Merrett (1993) reported that very high levels of NO₃ (>100 µM) can inhibit calcification and photosynthetic carbon fixation in highly calcifying strains. Merrett et al. (1993) demonstrated an absolute requirement of NO₃ for calcification, which may simply reflect the coupling between growth and calcification and the necessity of nitrogen for growth. A similar enhancement was recorded by Balch et al. (1992) in natural blooms as well as in laboratory batch cultures enriched by moderate NO₃ concentrations. Using NO₃-limited continuous cultures, Paasche (1998) showed that nitrogen limitation decreases the cellular organic carbon, the calcium content, and the size of coccoliths, but that it increases the number of coccoliths per cell, with a higher Ca/POC ratio at lower growth rates. In batch culture experiments, Berry et al. (2002) indicated that calcification is stimulated by decreased N concentration, and that, under low nutrients, increasing DIC can increase the rate of calcification, this effect being more pronounced at high pH (low pCO₂). Under low nutrient conditions, the absolute rate of photosynthesis was, as expected, significantly reduced. However, the increased C/P ratio in low N cultures reflected increased absolute rates of calcification, and not simply inhibition of photosynthesis. Our results show that the transition from unlimited batch to limited continuous-culturing mode was paralleled by an important decrease in the mean cell volume in both cultures (Fig. 2b), probably concomitant with a decrease in the carbon content (unfortunately not measured during the batch mode of this experiment).

The ratio of the PIC to POC production (or C/P ratio) has been shown to vary between 0 to 1.7 for non-calcifying and highly calcifying strains, respectively, with a modal value around 1 (see review by Paasche 2002).

The nature of these variations is not always easy to explain, since they involve multiple causes (strain origin, nutrient limitation, growth conditions, experimental artifacts) which may act separately or in combination. One of these causes is also the variation in DIC system-speciation which has been intensively studied by Buitenhuis et al. (1999). Riebesell et al. (2000) have shown that the C/P ratio in N-sufficient cultures of *Emiliana huxleyi* is decreased by 20% when pCO₂ is increased from 100 to 750 ppm by acid addition. Their Fig. 1 indicates that the reduction in the C/P ratio was due both to a stimulation of POC production and an inhibition of PIC production, although the former did not seem significant for pCO₂ ranging between 400 and 700 ppm. Their data (obtained in a subarctic North Pacific zone dominated by coccolithophorids) showed a similar effect of pCO₂ on the C/P ratio of natural communities but, in contrast with their laboratory experiment, they noted a marked (although non-significant) decrease in organic carbon production with increasing levels of pCO₂. In similar laboratory experiments conducted at various light intensities and non-limiting nutrient levels, Zondervan et al. (2002) observed similar responses of *E. huxleyi* to an augmentation of pCO₂, with systematic opposite variation in PIC and POC production, except at the lower irradiance used.

In agreement with the studies cited above, our data show that net cell calcification can be significantly and rapidly reduced following an increase of the pCO₂ in the medium. However, in contrast with the previous reports, in our study this effect was not accompanied by a significant decrease in the PIC/POC ratio, since POC production decreased roughly in proportion to PIC production. Considering that all experimental conditions were similar, the main difference between our study and the previous experiments lies in the fact that growth was nitrogen-limited in our study.

The question arises as to why an increase in pCO₂ results in a decrease in the net photosynthetically fixed carbon only when cells are nitrogen-limited. Inorganic carbon uptake, carbon fixation and respiration may be involved. Regarding respiration, several authors have reported that this can be affected in *Emiliana huxleyi* under pH conditions that differ from the 'standard' value of 8.0 (Paasche 1964). In the present experiment, the respiration rates were not significantly different between the control LL culture (pH = 8.02) and the pCO₂-increased HL culture (pH = 7.82), and was not significantly lower in the LH culture after CO₂-enrichment.

Sources of DIC for growth by calcifying strains of *Emiliana huxleyi* are CO₂ and HCO₃⁻. Despite the probable existence of a functional carbon-concentrating mechanism (CCM) (Nimer & Merrett 1996, Rost et al. 2003), the apparent affinity of *E. huxleyi* for DIC

remains low, so that photosynthesis in this species is considered by many authors to be non-carbon-saturated under conditions of $p\text{CO}_2$ commonly found in the ocean. Despite the lack of evidence for a protein associated with an active transport of CO_2 and HCO_3^- across the plasmalemma, external and internal carbonate anhydrase (CA) activities, generally associated with the CCM, have been detected in various strains of *E. huxleyi* (Nimer et al. 1994b, Elzenga et al. 2000, Rost et al. 2003). These activities are generally weak compared with activities in other species. The respective contributions of CO_2 and HCO_3^- to photosynthesis are controversial, depending on the uncertain importance of calcification as a source of H^+ and CO_2 for photosynthesis. Based on the assumption that the CO_2 and HCO_3^- uptake systems are regulated independently from each other, and that changes in pH have no significant effect on uptake kinetics, Rost et al. (2003) recently estimated that, at $p\text{CO}_2$ higher than 360 ppm, CO_2 remains the major substrate for carbon uptake in *E. huxleyi*. These findings are in contrast to those of Buitenhuis et al. (1999), who showed that CO_2 supports a modest rate of photosynthesis, whereas HCO_3^- is the major substrate for photosynthesis via the intracellular production of CO_2 by calcification ($\text{HCO}_3^- + \text{H}^+ \rightarrow \text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{CH}_2\text{O} + \text{O}_2$). In this scheme, HCO_3^- contributes significantly to the regeneration of CO_2 in the vicinity of RUBISCO, by providing H^+ via calcification in the coccolith-forming vesicle that (associated in the chloroplast with another HCO_3^- by a CA enzyme) yields CO_2 . Both the CCM and calcification are CO_2 down-regulated. Rost et al. (2003) showed that the DIC half-saturation constant for photosynthetic O_2 evolution may be decreased to some extent by increasing $p\text{CO}_2$ during growth. Similarly, the observed decrease in calcification after an increase in CO_2 has been interpreted as a greater ability of the cells to directly use external diffusing CO_2 for photosynthesis. Whatever the processes involved in DIC incorporation, they are not independent of nitrogen status. For instance, the activities of DIC transporters or other proteins involved in the CCM may decrease if nitrogen is limiting their synthesis (Beardall & Giordano 2002). Similarly, calcification implicates active transport of H^+ , HCO_3^- and Ca^{2+} through cell and coccolith vesicle membranes; this entails energy and enzymatic mobilization, while nitrogen is also required for the synthesis of the coccolith matrix material including glucoproteins (Rambourg 1967, Klaveness 1976). On the other hand, the improvement of the efficiency of N utilization by CCMs is a well-established concept for several microalgae (Beardall et al. 1998), provided that the nitrogen allocated to the CCM components does not offset the nitrogen saved through the CCM (Raven et al. 1985). Increasing $p\text{CO}_2$ from 412 to 708 ppm in our experi-

ment changed the DIC speciation: CO_2 and HCO_3^- increased by 72 and 9% in the culture, respectively, whereas CO_3^{2-} decreased by 31%. The fact that the redistribution of the potential carbon substrates for growth (CO_2 and HCO_3^-) in the seawater could be responsible for a globally less efficient carbon uptake under conditions of nitrogen limitation is, in the framework of this study, a speculative question that requires further experiments on the energy costs involved in calcification and CCM (Anning et al. 1996). In this context, it can be hypothesized that if nitrogen limitation has a relatively greater depressive effect on CCM than on calcification, then carbon fixation would become more dependent on the CCM and should decrease in response to the decline in calcification driven by elevated $p\text{CO}_2$.

Besides carbon uptake and respiration, another possible cause for the observed decrease in net production is the lowering of internal pH, indirectly related to the fixation of carbon itself. Many species of marine phytoplankton restrict intracellular pH between 7 and 7.4 by ion-transport mechanisms and a high buffering capacity of the cytosol and, under N-replete conditions, these species show enhanced growth at elevated CO_2 concentrations (Beardall & Raven 1981). Fixation of carbon is ultimately under the control of RUBISCO activity, with a cell carboxylating capacity depending on the amount of this enzyme in the chloroplasts. Rubisco can contain up to 1/10th of the total nitrogen in a cell, and since it is a major reservoir of nitrogen in cells, it is not surprising that nitrogen limitation leads to a significant reduction in Rubisco and the reallocation of nitrogen resources. One of the direct consequences of a restricted carboxylase activity should be the accumulation of CO_2 not fixed by Rubisco. Should nitrogen-limited cells be unable to utilize the extra CO_2 produced at high ambient $p\text{CO}_2$, passive diffusion of CO_2 across the plasmalemma could result in acidification of the cytosol. This acidification should be larger in the HL than in the LL culture, given the higher concentration of CO_2 reached in the former after the $p\text{CO}_2$ shift. Nimer et al. (1994a) noticed that, at a constant external pH of 8.3, the internal pH of a highly calcifying strain of *Emiliana huxleyi* decreased from 6.77 to 6.38 when the volume ratio of CO_2/air bubbled was increased from 0.03 to 0.1%. Parallel to this effect, Nimer et al. (1994a) provided evidence for the negative effect of high $p\text{CO}_2$ on the growth rate and yield of *E. huxleyi*, which they partially attributed to the decrease in internal pH. This inhibition of growth rate by a high $p\text{CO}_2$ treatment could be related to the inability of *E. huxleyi* to generate sufficient OH^- to neutralize the H^+ produced by calcification (Nimer & Merrett 1993) and thus counteract the acidification of the cytosol.

Emiliana huxleyi has a very high affinity for phosphates (Egge & Heimdal 1994, Riegman et al. 2000) and a relatively low requirement for Fe (Brand et al. 1983, Muggli & Harrison 1996). It follows that nitrate, in addition to the DIC substrate, may control organic production. Our results show that, under conditions of nitrogen limitation in continuous cultures, the effect of pCO₂ increase on the C/P ratio differs from that observed in nutrient-replete cultures. This experiment was performed at only a moderate growth limitation of 0.5 d⁻¹. Further experiments, performed in a light-dark cycle, should be conducted to appreciate the differential effects of nitrogen limitation on the relationship between pCO₂ and the C/P ratio.

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