Botanica Marina Vol. 45, 2002, pp. 491–501

Growth and Biochemical Composition of the Diatom *Phaeodactylum tricornutum* at Different pH and Inorganic Carbon Levels under Saturating and Subsaturating Light Regimes

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The effects of pH and total dissolved inorganic carbon (DIC) availability on the growth and biochemical composition of *Phaeodactylum tricornutum* Bohlin acclimated to restricted and saturated light conditions have been investigated. Diluted, unbuffered and nutrient enriched batch cultures were grown at several pHs (different $[CO_{2(aq)}]$ for the same DIC) and various DIC conditions (different $[CO_{2(aq)}]$ for an equal pH) under saturating (150 μmol m^{-2} s^{-1}) and subsaturating (30 μmol m^{-2} s^{-1}) light levels. The growth rate was unaffected by pH treatments at saturating light. In contrast, under subsaturating light conditions, the growth rate was significantly reduced at pHs over 8.5 and also at low DIC levels to a similar extent. Biochemical composition revealed that the growth rate was limited by different resources. At natural DIC (DIC = 2.1 mM) and low light conditions, there was an increase of the C:N ratio under high pH (low $[CO_{2(aq)}]$) associated with a reduction of nitrogen, chlorophyll *a* and total protein content, resembling the biochemical composition of a diatom cultured under nitrogen limitation. On the contrary, under low light and low DIC conditions, in which both $[CO_{2(aq)}]$ and $[HCO_3^-]$ are reduced, cellular carbon content decreased for a relatively constant nitrogen content, decreasing the C:N ratio. The results are discussed in terms of competition between carbon and nitrogen metabolism for energy, and showed that the imposition of different $[CO_{2(aq)}]$ by changing pH or DIC levels of seawater affected in different ways the final biochemical composition of *Phaeodactylum tricornutum*.

Introduction

Natural seawater contains a total dissolved inorganic carbon (DIC) concentration of around 2 mM. Most of this carbon (95 %) is in the form of bicarbonate anion, with an equilibrium concentration of $CO_{2(aq)}$ of only 10 μM, at 18 °C and pH of 8.2. Under optimal light and nutrient conditions, the acquisition of such relatively scarce $CO_{2(aq)}$ has been proposed as a potential limiting step for photosynthesis and growth in marine phytoplankton (Riebesell *et al.* 1993, Chen and Durbin 1994). This hypothesis has led to an increase in the biochemical and physiological studies centred on the definition of the mechanisms for carbon acquisition in marine microalgae, and also in the form of inorganic carbon mainly taken up by phytoplankton (Raven and Johnston 1991, Korb *et al.* 1997, Nimer *et al.* 1997, Tortell *et al.* 1997, Reinfelder *et al.* 2000). Diatoms which play a predominant role in primary productivity of coastal and oceanic waters have been intensively investigated, although most of the studies have been focused on a limited number of genera and species. *Phaeodactylum tricornutum* Bohlin has been one of the more extensively studied species. Notwithstanding that, there have been conflicting reports in the literature about carbon acquisition mechanisms, as well as on the location of the carbon concentrating mechanism in this species. An active Na^+/HCO_3^- symport through the plasmalemma (Patel and Merrett 1986, Dixon and Merrett 1988), bicarbonate use associated with an external carbonic anhydrase (CA) (Burns and Beardall 1987), an active transport of HCO_3^- not associated with Na^+ (Colman and Rotatore 1995) or an active uptake of CO_2 (Rotatore *et al.* 1995) have all been suggested as mechanisms for carbon acquisition in this species. In spite of the energetic requirement for most of the mechanisms mentioned above, there are no published data about the effects of subsaturating light conditions, commonly found in nature, on carbon acquisition in this diatom. Light should play an important role in the efficiency of inorganic carbon acquisition mechanisms (optimal activity of implicated enzymes, ATP availability for active carbon uptake, etc.) and, consequently, its restriction may affect the final carbon quota, cellular composition and the growth rate. The studies centred on growth and biochemical composition of *Phaeodactylum tricornutum* have embraced variations in response to different environmental conditions, such as light (e.g. Geider *et al.* 1986, Thompson *et al.* 1991, Chrismadha and Borowitzka 1994), and nutrient (nitrogen and phosphorus)

availability (Siron *et al.* 1989, La Roche *et al.* 1993, Larson and Rees 1996, Otero *et al.* 1998), but reports on the effect of CO_2 and/or DIC availability are scarce. Burkhardt *et al.* (1999) have shown the constancy of elemental composition (C:N:P) and growth rates of *P. tricornutum* cultured under saturating light in a range of $[CO_{2(aq)}]$ from 1.5 to 37.7 μmol Kg^{-1} but there are not equivalent results for subsaturating light conditions. Thus, acclimation mechanisms for carbon acquisition and biochemical responses to carbon or light availability are frequently regarded as unrelated topics. However, both aspects, light and inorganic carbon, should be considered when studying the effect of different inorganic carbon availability on the algal physiology. With the aim of studying the combined effects of different light and carbon levels on growth and biochemical composition of *Phaeodactylum tricornutum* we used the strain CCAP 1052/1A, whose most accepted mechanism for inorganic carbon acquisition seems to be an active bicarbonate transport that increases the cell's apparent affinity for CO_2 (John-McKay and Colman 1997).

In theory, if *P. tricornutum* is able to maintain a constant growth in a wide range of $[CO_{2(aq)}]$ by active uptake of bicarbonate the effect of light (energy) reduction on growth rate under CO_2 or DIC limitation should be notable, as well as the effect on biochemical composition. According to the above hypothesis, the objectives of this study were: 1) to compare the combined effects of inorganic carbon and light availability on the growth rate of *P. tricornutum* (strain CCAP 1052/1A), 2) to analyze the biochemical alterations of this diatom associated with changes in CO_2 levels, obtained by altering pH or altering total inorganic carbon concentrations in the media, at saturating and subsaturating growth light levels, and 3) to discuss the physiological and ecological implications of light reduction on cell acclimation to different inorganic carbon conditions.

Material and Methods

Culture conditions

The marine diatom *Phaeodactylum tricornutum* (clone CCAP 1052/1A), obtained from the Alfred Wegener Institute culture collection, was maintained in stock culture in filtered (0.2 μm) seawater (Gulf of Cádiz, Southern Spain) at 17.5 ± 0.5 °C under saturating and continuous white light (fluorescent lamps, Sylvania GRO-LUX F36W-GRO T8, Sylvania, Erlangen, Germany). Experiments were performed in sterilized 1-L borosilicate glass bottles. To ensure there was no nitrogen or phosphorus limitation during growth, 0.2 μm filtered natural seawater was enriched with f/2 medium (Guillard and Ryther 1962). Unbuffered media were used in all the experiments, since some authors have shown that the use of buffers can affect the physiology of several diatom species, including *P. tricornutum* (Fábregas *et al.* 1993, Blanchemain *et al.* 1994).

Experimental treatments were set up by the combination of different initial pH and dissolved inorganic carbon (DIC) levels (Table I) under two light

Table I. Experimental carbonate system.

Treatments	pH	$[CO_{2(aq)}]$ (μM)	DIC (mM)
		Saturating light	
pH 7.9–natural DIC	7.96 (17.2)	24.8 ± 0.6	2.20 ± 0.03
pH 8.2–natural DIC	8.19 (17.5)	13.8 ± 0.1	2.20 ± 0.01
pH 8.5–natural DIC	8.49 (17.2)	8.0	2.10
pH 8.9–natural DIC	8.89 (17.5)	2.0 ± 0.0	2.22 ± 0.01
pH 8.2–high DIC	8.19 (18.6)	23.3 ± 1.2	3.75 ± 0.15
pH 8.2–natural DIC	8.19 (17.5)	13.8 ± 0.1	2.20 ± 0.01
pH 8.2–low DIC	8.19 (17.5)	7.1	1.14
		Subsaturating light	
pH 7.9–natural DIC	7.91 (17.8)	22.5 ± 0.9	2.07 ± 0.01
pH 8.2–natural DIC	8.19 (17.5)	11.4 ± 0.9	1.94 ± 0.01
pH 8.5–natural DIC	8.49 (17.5)	6.0	2.10
pH 9.0–natural DIC	8.88 (17.7)	2.2 ± 0.1	2.25 ± 0.01
pH 9.5–natural DIC	9.50 (17.4)	0.2 ± 0.0	2.00 ± 0.00
pH 8.2–high DIC	8.20 (18.7)	21.7	3.70 ± 0.03
pH 8.2–natural DIC	8.19 (17.5)	11.4 ± 0.9	1.94 ± 0.01
pH 8.2–low DIC (1)	8.20 (17.6)	9.4 ± 1.0	1.52 ± 0.02
pH 8.2–low DIC (2)	8.19 (17.2)	2.9 ± 0.4	0.38 ± 0.01

Most of the values are the means ± SD of different cultures with equivalent conditions (n = 4). Temperature (°C) for pH measurements is indicated in brackets.

growth regimes, 150 and 30 μmol m^{-2} s^{-1}, which are considered to be saturating and subsaturating growth light levels respectively for this diatom (Beardall and Morris 1976), at 14:10, L:D cycles. Temperature ranged between 17.5 ± 0.5 °C during the light phase and 15.5 ± 0.5 °C during the dark phase. Cells were grown at both light intensities in two different types of media: 1) In seawater with natural DIC concentration (2.1–2.2 mM) and different initial [$CO_{2(aq)}$], obtained by changing the pH of the media to initial values of 7.9, 8.2 (natural), 8.5 and 8.9 by adding 1-N solutions of HCl or NaOH. In the case of subsaturating light conditions, pH 9.5 was also assayed. The selected pHs allowed us to embrace a wide range of [$CO_{2(aq)}$] for an equal DIC (Table I). 2) In seawater with natural pH (8.2) and different DIC levels (and consequently different [$CO_{2(aq)}$]). High DIC media were prepared by adding $NaHCO_3$ to the natural seawater reaching a final DIC concentration over 3 mM. In these media the pH was checked to be 8.2 after adding $NaHCO_3$. Natural DIC media consisted of natural seawater, that is DIC = 2.1–2.2 mM. Low DIC media were prepared by bubbling with air that has passed through acidified seawater (HCl 1N) and adjusting the final pH to a value of 8.2 by adding 1 N NaOH. After the preparation of the media, bottles were immediately closed without any headspace to avoid [CO_2 (g)] diffusion that could alter the pre-established pH and [$CO_{2(aq)}$].

Experimental design

The experiments were designed to ensure the acclimation of the cells to the different inorganic carbon conditions but preventing a strong alteration of the carbonate system, which is affected by cell growth in unbuffered media. Consequently, low cell density cultures were used (300–400 cell mL^{-1} at day 0), and the pH was employed as a parameter for monitoring the carbonate system conditions since an increase of 0.1 unit from the pre-established pH in the cultures would be indicative of a complete loss of the desired carbon conditions (as an example, an increase of 0.1 units of pH implies a decrease of [$CO_{2(aq)}$] from 12.5 to 9.4 μM for a seawater of 38 psu, 2.2 mM of DIC, pH 8.2 and 17.5 °C). Cells were pre-acclimated to the different experimental conditions for at least eight cell divisions (4–6 days). Thus, for each treatment (Table I), 15 sterile 1.2-L borosilicate glass bottles were filled with the same culture medium and inoculated with an identical low cell density from the above mentioned pre-acclimated culture. Bottles were turned over 3 times during the light phase to keep the cells in suspension. The pH was continuously monitored in one of these bottles (considered as a reference bottle) with a pH-meter model CRISON-2002 (Crison Instruments S.A., Alella, Spain) using a combined AgCl/KCl glass electrode (CRISON 50–02) calibrated with National Bureau Standards (NBS) buffer solutions. Daily, two of the remaining 14 bottles were randomly withdrawn for cell counting and carbonate system monitoring, as will be explained below. These bottles were not further incubated. There was a coincidence of the discrete (two bottles per day) and the continuous (reference bottle) pH profiles during the experimental period and consequently the different bottles were considered as daily samples of an equivalent culture. When culture biomass was high enough to ensure reliable biochemical quantifications, but the carbonate system was still largely unaffected by cell growth (pH values never increased 0.1 units from the pre-established pH), the two bottles used for cell counting and inorganic carbon analyses were also used for the quantification of cellular carbon (C/cell), cellular nitrogen (N/cell), total proteins (TP/cell), total carbohydrates (TCH/cell) and chlorophyll *a* (Chl *a*/cell) contents. These dates ranged between the fourth day of growth for high light acclimated cells to the sixth day of growth for the slowest growing low light acclimated culture. To avoid photoperiod effects on biochemical composition, bottles were always withdrawn at the same hour, 7 h after the onset of the light.

Chemical analyses of the carbonate system

To estimate the [$CO_{2(aq)}$] in the cultures, DIC, total alkalinity (TA), salinity, phosphate and silicate concentrations were measured daily. Duplicate samples of 15 mL per bottle were taken for DIC measurements. Samples were fixed with a saturated $HgCl_2$ solution, and stored in sterile Pyrex tubes in the dark and without headspace until analysis. The DIC was quantified using a total organic carbon analyzer TOC-5050 (Shimadzu, Tokyo, Japan) measuring the CO_2 after the acidification of the samples (50 %-H_3PO_4) by non-dispersive infra-red analysis. The TA was measured by titration technique according to Gran (1952) using a Metrohm 713-pH meter (Metrohm AG Company, Herisau, Switzerland) connected to an automatic burette 665 Dosimat (Metrohm AG Company, Herisau, Switzerland). Duplicate samples of 100 mL per bottle were titrated by adding 0.1 mL increments of a 0.05 N HCl + NaCl 35 g L^{-1} solution in a thermostated cell (20 °C ± 0.1). Salinity was measured with an induction salinometer (Model RS10 Rosemount Analytical Inc., Cedar Grove, NJ, U.S.A.) calibrated with a KCl solution (23.44 g L^{-1}). Phosphate and silicate concentrations were also quantified for carbonate calculations. Duplicates of 10 mL per bottle were filtered through 0.45 μm Millipore filters and stored frozen until analysis. Measurements were performed by a Bran+Luebbe TRAACS 800 (Bran+Luebbe Inc., Norderstedt, Germany) analyzer according to Grasshoff *et al.* (1983). The carbon speciation was calculated using carbonic acid dissociation constants of Merhbach *et al.* (1973).

Cell concentration and growth rates

From the two bottles selected daily, duplicate subsamples of 125 mL per bottle were collected and fixed with Lugol's solution. These subsamples were kept in a cold dark place until analysis. Cell number was counted using an inverted microscope (Utermöhl 1958). A minimal number of 400 cells per replica were counted in order to keep the counting error within ± 10 % (Lund *et al.* 1958). Growth rates were calculated according to an exponential model.

Bacterial biomass was checked to be less than 10 % of phytoplankton biomass. Samples were preserved in 0.6 % glutaraldehyde and stored at 4 °C in the dark. Bacterial biomass was quantified using epifluorescence microscopy with 4', 6 diamidino-2-phenylindole (DAPI) as fluorochrome (Porter and Feig 1980). Organisms were concentrated on to 0.2 µm pore-sized polycarbonate filters (Poretics) using a Leitz Laborlux epifluorescent microscope (Wild Leitz Mikroskopie und Systeme GmbH, Wetzlar, Germany).

Biochemical composition

Triplicates of 10 mL per bottle were taken to determine Chl *a* concentration. Samples were concentrated by filtration through Whatman GF/F glass fiber filters, placed in 8 mL of 90 % acetone and stored at 4 °C in the dark for 12 h. The Chl *a* was quantified fluorometrically (UNESCO 1994), using a fluorometer (Model 10–005 R, Turner Designs, Sunnyvale, U.S.A.) calibrated with Chl *a* from *Anacystis nidulans* (SIGMA C-6144).

The C/cell and N/cell were determined by filtering a variable (50–100 mL) volume at low vacuum (< 100 mM Hg) on precombusted (5h–450 °C) GF/F glass fibre filters (Whatman), rinsed three times with 5 mL of 0.17 M Na_2SO_4 and stored at –20 °C until analysis (Burkhardt and Riebesell 1997). Particulate samples on GF/F filters were exposed to gaseous concentrated HCl to remove carbonates. Quantification was performed on duplicate filters on a Perkin-Elmer 240-C elemental analyzer (Perkin Elmer ™ Analytical Instruments, Shelton, U.S.A.).

Duplicate samples per bottle (50–75 mL) for total carbohydrates (TCH) analyses were filtered through precombusted (5 h–450 °C) GF/F glass fibre filters and stored at –20 °C until analysis. The TCH were quantified using the phenol-sulfuric method (Dubois *et al.* 1956) following the protocol described by Fisher and Harrison (1996). Total protein content (TP) was quantified on duplicate samples per bottle, concentrated by filtration (50–75 mL) on polycarbonate filters (Millipore), ground in 10 % w/v trichloroacetic acid-acetone (TCA-acetone), sonicated and stored at –20 °C for 1 h. After centrifugation (15,000 rpm) the pellet was sonicated again and redissolved in 100 % acetone. Extracts were centrifuged (15,000 rpm) and the acetone was discarded. The TP contained in the dried pellet were extracted in 100 µL of carbonate buffer (0.2 M Na_2CO_3-4 % sodium dodecylsulfate, SDS) and stored at –80 °C until analysis. The TP content was determined by the colorimetric method of Smith *et al.* (1985) using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as standard (Fernández *et al.* 1992). Assays were run on a THERMOmax® microplate reader (Molecular Devices Corporation, Menlo Park, U.S.A.) using DeltaSOFT® software version 3.0 (Molecular Devices Corporation, Menlo Park, U.S.A.).

Statistics

Statistical analyses (one-way ANOVA) and post-hoc Tukey tests were applied (Fry 1993). The significance levels were set up at $p < 0.05$. A slope comparison t-test (Zar 1984) was used to analyse differences ($p < 0.05$) between growth rates.

Results

Growth rates

Growth rates were light limited at 30 µmol m^{-2} s^{-1} at all conditions assayed. At this subsaturating irradiance, growth rates of *Phaeodactylum tricornutum* showed a significant reduction at pH values above 8.5 ($p < 0.05$), that is, when $[CO_{2(aq)}]$ was low (Fig. 1A). At low light, there were also significant differences when comparing growth rates at different DIC levels ($p < 0.05$; Fig. 1B), which means different $[CO_{2(aq)}]$ at the same pH. In contrast, growth rate was not affected by carbon availability ($\mu = 1.35 \pm 0.08$ d^{-1}) under light saturated conditions (150 µmol m^{-2} s^{-1}) in both types of media.

Biochemical composition

The C/cell was not significantly affected ($p > 0.05$) by pH at both light intensities (Fig. 2A). However, N/cell was significantly reduced ($p < 0.001$) at pHs over 8.9 (or $[CO_{2(aq)}] \leq 2.1$ µM) (Fig. 2B) under subsaturating light level. Consequently, under low light conditions, the C:N ratio increased for the lowest $[CO_{2(aq)}]$ (Fig. 2C) coincident with lower growth rates. In the cultures acclimated to pH 8.2 and different DIC conditions, C/cell was fairly constant in high light acclimated cells at all conditions assayed, but decreased significantly ($p < 0.0001$) towards lower DIC in low light acclimated cells (Fig. 2D). The N/cell was not altered at low DIC (LDIC) levels for either irradiance (Fig. 2E) although, under high DIC (HDIC) conditions, N/cell decreased at high irradiance. The resulting C:N ratio was extremely low for LDIC and low light acclimated cells (Fig. 2F), which will be discussed later. For the DIC enriched medium, the C:N ratio was higher than at natural DIC conditions at both irradiances.

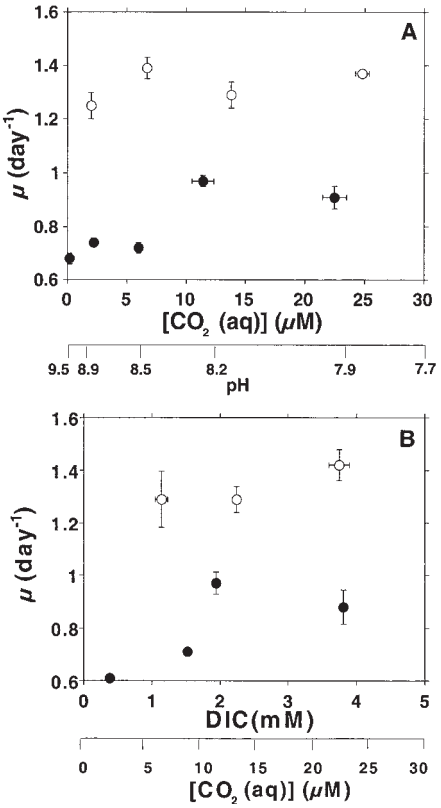
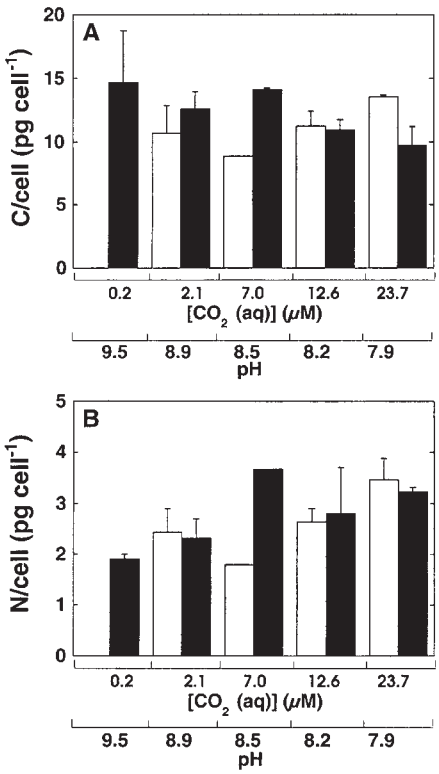


Fig. 1. Growth rates (μ) of *P. tricornutum* acclimated to saturating (open circles) and subsaturating (closed circles) growth irradiances as a function of A) $[CO_{2(aq)}]$ in cultures acclimated to different initial pHs and B) DIC in cultures acclimated to different initial DIC levels for a constant pH of 8.2.



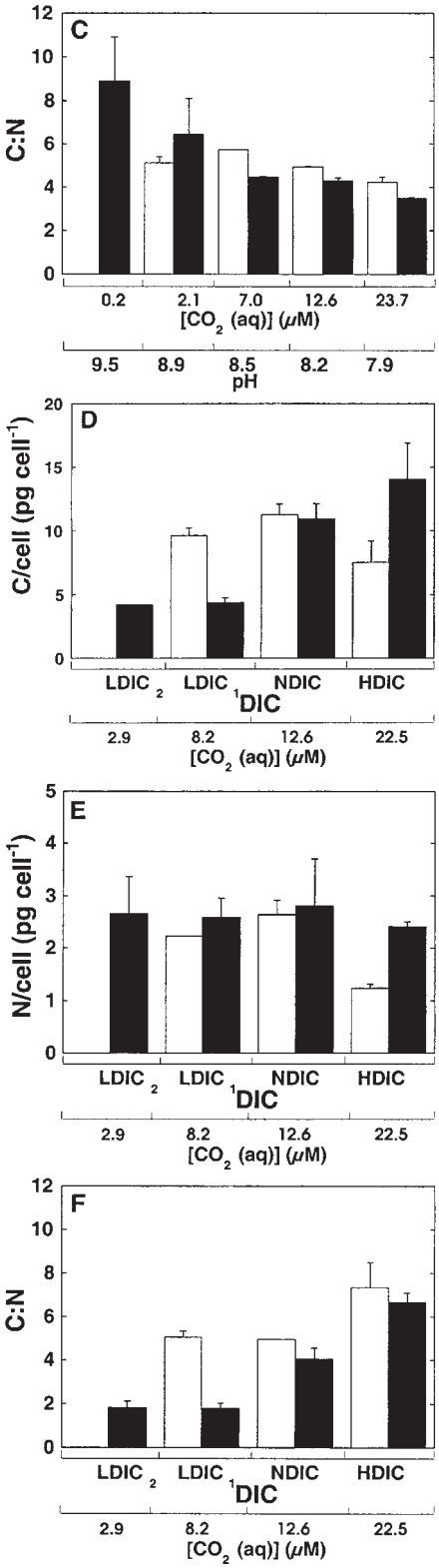


Fig. 2. C/cell (pg cell^{-1}), N/cell (pg cell^{-1}) and C:N ratio (by atoms) of *P. tricornutum* cultured at saturating (white bars) and subsaturating (dark bars) light levels at different initial $[CO_{2(aq)}]$ (different pH) (A, B and C) or different initial DIC levels (D, E and F). HDIC: high DIC level; NDIC: natural DIC level; LDIC: low DIC level. Concentrations indicated on Table I. The levels of CO_2 indicated on the x-axis are the mean of the $[CO_{2(aq)}]$ obtained for saturating and subsaturating light conditions (see Table I).

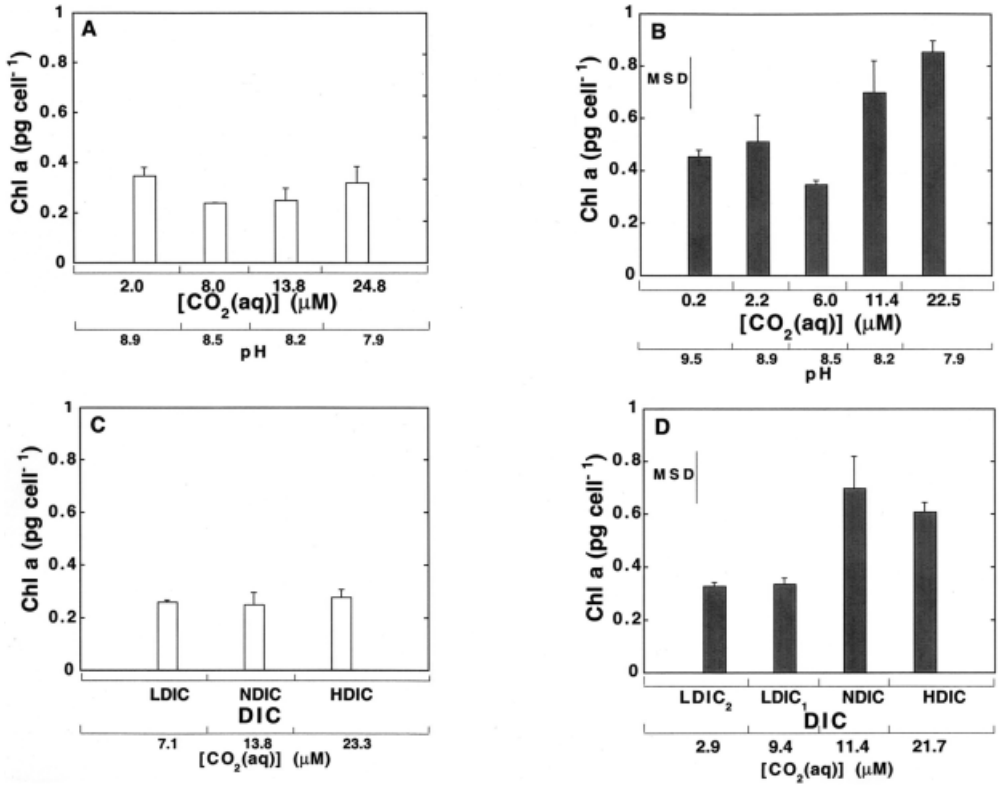


Fig. 3. Chlorophyll *a* content (pg cell^{-1}) of *P. tricornutum* grown at 150 μmol m^{-2} s^{-1} (white bars) and 30 μmol m^{-2} s^{-1} (dark bars) at different initial [$CO_{2(aq)}$] (different pH) (A and B) and different initial DIC levels (C and D). MSD: minimum significant difference. Abbreviations of DIC levels as those in Figure 2.

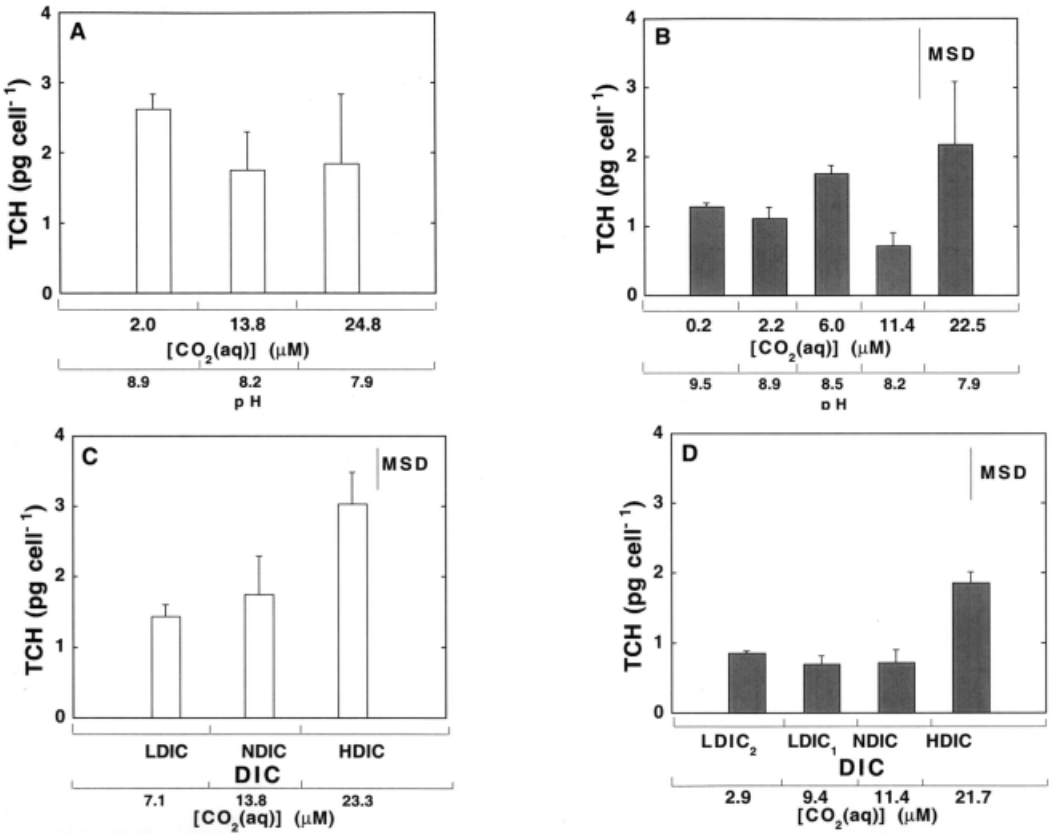


Fig. 4. Total carbohydrates (TCH) content (pg cell^{-1}) of *P. tricornutum* grown at 150 μmol m^{-2} s^{-1} (white bars) and 30 μmol m^{-2} s^{-1} (dark bars) at different initial [$CO_{2(aq)}$] (different pH) (A and B) and different initial DIC levels (C and D). MSD: minimum significant difference. Abbreviations of DIC levels as those in Figure 2.

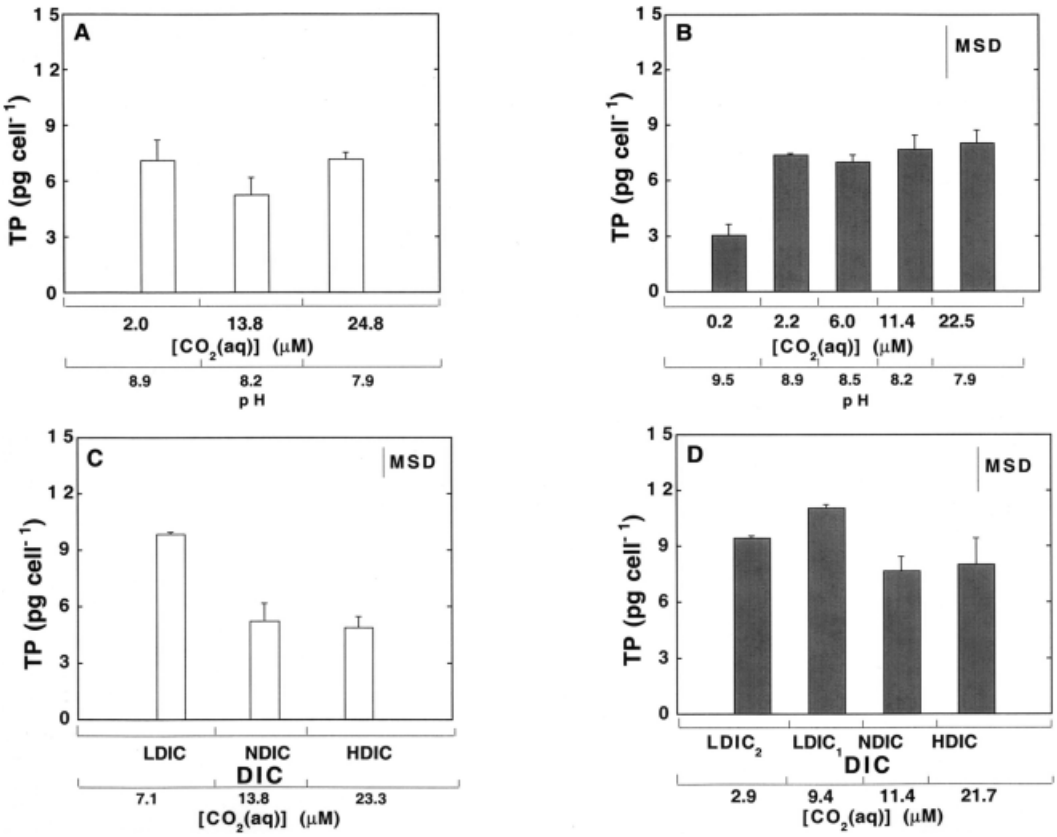


Fig. 5. Total proteins (TP) content (pg cell^{-1}) of *P. tricornutum* grown at 150 μmol m^{-2} s^{-1} (white bars) and 30 μmol m^{-2} s^{-1} (dark bars) at different initial [$CO_{2(aq)}$] (different pH) (A and B) and different initial DIC levels (C and D). MSD: minimum significant difference. Abbreviations of DIC levels as those in Figure 2.

Light intensity affected Chl *a* content in *Phaeodactylum tricornutum* (Fig. 3). Low light acclimated cells showed higher Chl *a* content than high light acclimated ones in most of the treatments assayed. There were no significant differences ($p > 0.05$) in the Chl *a* content of high light acclimated cells at different pH or different DIC conditions (Fig. 3A and C). However, at subsaturating light conditions, Chl *a* content was significantly reduced at high pH (low [$CO_{2(aq)}$]) (Tukey-Kramer test; $p < 0.0001$) (Fig. 3 B), and low DIC acclimated cells (Fig. 3 D).

The TCH content in *P. tricornutum* was influenced by light intensity (Fig. 4). Overall, high light acclimated cells showed a higher TCH content than low light acclimated ones. Under saturating light conditions the TCH content was not dependent on initial [$CO_{2(aq)}$]or pH (Fig. 4A). However, at low light conditions, TCH was significantly affected by pH although a clear pattern was not observed ($p < 0.0001$; Fig. 4 B). Cells acclimated to high DIC media increased TCH significantly at both light intensities ($p < 0.0001$; Fig. 4 C and D).

The TP content varied significantly ($p < 0.0001$) in cells acclimated to different pHs at low light, but was much less variable under saturating light levels (Fig. 5A and B). Under subsaturating light conditions, cells acclimated to the lowest [$CO_{2(aq)}$] showed the lowest TP content (Fig. 5B). Cells acclimated to low DIC availability conditions increased the TP content significantly ($p < 0.0001$) at both light intensities (Fig. 5C and D) in comparison with natural DIC acclimated cells.

Cellular carbon and nitrogen production rates

Under subsaturating light conditions, in which growth rates were affected by carbon experimental treatments, the biomass (carbon and nitrogen) produced per cell and unit of time was estimated (Fig. 6). It was estimated by using carbon and nitrogen cell quota (pg cell^{-1}) and the generation time (T_g = Ln2/μ). Under different pH conditions, carbon production rates were equivalent in all treatments, but nitrogen ones decreased for pH > 8.5, coinciding with lower growth rates (Fig. 6A). On the contrary, cells grown under pH 8.2 and different DIC levels showed the reverse trend, with similar production rates for nitrogen but a lower production rate for carbon in LDIC treatments (also, lower growth rates) (Fig. 6B).

Discussion

The results presented here show that under saturating light levels the growth rate of *Phaeodactylum tricornutum* remained relatively constant at different pHs

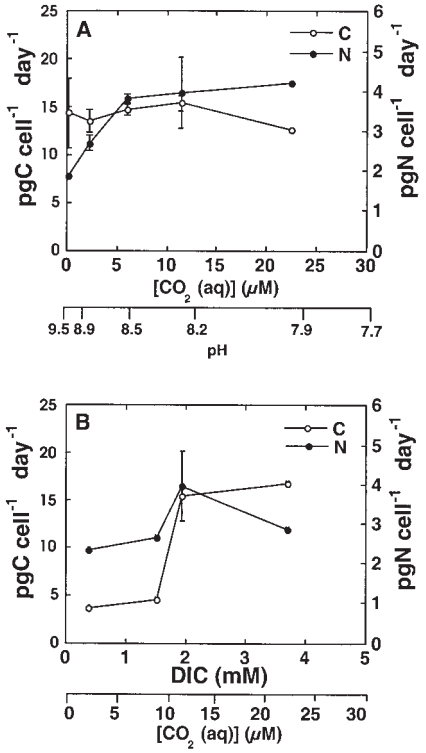


Fig. 6. Cellular carbon and nitrogen production rates, obtained from carbon and nitrogen cell quota (pg cell^{-1}) and division (generation) time (T_g = days), of *P. tricornutum* grown under subsaturating irradiance at different pHs (A) and different initial DIC levels (B).

$[CO_{2(aq)}]$ from 24 to 2 μM), with values of around 1.32 d^{-1} (Fig.1A). This finding had been previously shown by Burkhardt *et al.* (1999). In addition, and for the same light regime, the DIC level during growth did not seem to affect the capacity of cells for growth since μ was found to keep similar values to those in the previous treatment (Fig.1 B). The growth response at high irradiance was reflected in the biochemical composition of the cells, since C and N cell contents were slightly affected by carbon availability. These results can be explained through the occurrence of a bicarbonate uptake mechanism in this strain that would become active under low $[CO_{2(aq)}]$ (Patel and Merrett 1986, Dixon and Merrett 1988, Colman and Rotatore 1995, John-McKay and Colman 1997), allowing the cells to acclimate to different CO_2 concentrations.

On the contrary, at subsaturating irradiance the growth rate decreased at high pH and also at low DIC conditions (low CO_2 in both cases) to a similar extent (Fig. 1A and B) and was accompanied by relevant changes in the biochemical composition of cells. Since the cells were grown under a L:D cycle, dark respiration could be responsible for such growth and biochemical alterations. Nonetheless, no significant variations of the pH/CO_2 profiles during darkness, which can be considered as an indirect estimation of dark respiration rates, were detected (not shown) for the studied treatments.

Changes in biochemical composition, such as the increase in C:N values and the reduction in N/cell content at pHs over 8.5, are indicative of a lower efficiency of nitrogen acquisition for a relatively unchanged carbon production (Fig.6). A comparable increase in the C:N ratio has been observed in the haptophyte *Isochrysis galbana* Parke as a response to N stress (Clark *et al.* 1999). Moreover, the reduction in Chl *a* content associated with these conditions has been described in nitrogen limited microalgae (Osborne and Geider 1986, Falkowski *et al.* 1989). Since the semisaturation constant for NO_3^- ranges from 0.1 to 10 μM for *Phaeodactylum tricornutum* (Collos 1980) and cultures were enriched with 2 mM nitrate, the possibility of nitrogen limitation during growth was highly unlikely to occur. However, the competition between both nitrogen and carbon metabolisms for photosynthetic ATP, NADPH and carbon skeletons could be considered as a plausible explanation for such a response, especially under subsaturating irradiance. In fact, *P. tricornutum* possesses an active Na^+-dependent nitrate transport (Rees *et al.* 1980) which would compete, in terms of energy requirement, with the HCO_3^- transporter. Based on the final biochemical composition observed (higher C/cell and lower N/cell towards higher pH, Fig. 2), it can be suggested that the energy would have been preferentially used to support the inorganic carbon uptake in contrast to nitrogen acquisition. At saturating light, the energy available to drive both nitrate and inorganic carbon acquisitions is expected to be sufficient to satisfy the energy requirement and consequently, the C:N ratio, TCH and TP maintained irrespective of the available $[CO_{2(aq)}]$.

Considering that the interdependence of the carbonate system by changes of $[CO_{2(aq)}]$ makes necessary the alteration of at least one of the parameters DIC, pH or alkalinity, then the question arises whether there is a 'pure' pH effect on elemental composition and growth rate, or if the alterations observed are a consequence of exclusively low $[CO_{2(aq)}]$. Regarding the pH, it is interesting to point out that high pH values and subsaturating light conditions have been shown to inhibit ammonium and nitrate uptake in microalgae (Zevenboom 1986). In addition, silicate incorporation in *P. tricornutum* is also dependent upon the pH during growth (Del Amo and Brzezinski 1999). Thus, the pH itself could have had a decisive influence on the nitrate uptake. Recently, a decrease of growth rates in the diatom *Skeletonema costatum* Cleve at pH values over 9 has been attributed to a pH effect (Taraldsvik and Myklestad 2000). Moreover, it is important to consider that substrate and enzyme exist in different ionic forms being dependent on pH and Michaelis-Menten parameters (K_m and V_{max}), respectively (Vega-Catalán 1990). Consequently, DIC incorporation rates, as other nutrients, can vary as a function of proton concentration in the media, since the protonation

and deprotonation of the active site of enzymes is the main cause for alteration of such incorporation rates with pH (Vega-Catalán 1990). The mechanism of inorganic carbon acquisition present in *Phaeodactylum tricornutum* should be taken into account at this point. Under low [$CO_{2(aq)}$], the bicarbonate uptake system would transport actively HCO_3^- which would have to be converted to CO_2 inside the cell for photosynthetic fixation. This implies an extra demand of [H^+] to allow the bicarbonate protonation to proceed. Simultaneously, protons would be also required in order to maintain the intracellular pH (Raven 1990). Such a high demand would be difficult to sustain in an alkaline medium and at subsaturating irradiance. Then, it is difficult to differentiate between low CO_2 and high pH, or both, as the main causes affecting growth and biochemical composition in *P. tricornutum* grown under subsaturating light, but, as our results are the first to document, it seems clear that these alterations are light dependent. Therefore, in addition to a competition for energy between carbon and nitrogen metabolisms under light restricted conditions, the effect of proton concentration affecting the nutrient incorporation routes at the level of enzymatic activity can not be ruled out.

On the other hand, changes in total DIC (from 3.7 mM to 0.4 mM) markedly determined the biochemical composition of *P. tricornutum*. In particular, the C/cell dropped from 14 pgC $cell^{-1}$ at high DIC to 4.4 pgC $cell^{-1}$ at low DIC, which was also accompanied by a reduction in growth rates. Since the abundance of the different inorganic carbon forms in seawater ultimately depends upon the total DIC, and at pH 8.2 most of it exists as bicarbonate, a diminution in the carbon content in low DIC acclimated cells (< 1.5 mM) might be correlated with a low bicarbonate availability in the medium. Low light acclimated cells showed a drastic reduction of carbon content under low DIC conditions whereas the cell nitrogen remained unchanged. This response entailed C:N ratios considerably lower than that predicted by Redfield stoichiometry but in the range that has been reported in spring diatom blooms growing on NO_3^- and different waterbodies (see Lomas and Glibert 2000). Similarly, a decrease in C/cell under inorganic carbon stress conditions has been described in *Thalassiosira pseudonana* Hustedt and *Thalassiosira weissflogii* Grunow cultured at saturated light conditions (Clark 2001). Low C:N assimilation ratios have also been related with accumulation of no nutritional inorganic nitrogen as a consequence of periods of imbalance between light energy harvesting and utilization (Lomas and Glibert 1999). The reduction of the accumulated NO_3^- may serve as a sink for electrons during such periods. According to the results obtained in *Phaeodactylum tricornutum* this imbalance could be a consequence of a very low DIC availability.

The results obtained in this work showed the light dependence of inorganic carbon acquisition under high pH (low [$CO_{2(aq)}$]) and low DIC conditions in *P. tricornutum*. We first report that equivalent reduction in growth rate does not correlate with similar alterations of cell composition when [$CO_{2(aq)}$] is changed by using either different pH values or DIC levels. This response is relevant not only physiologically but ecologically. Considering a present day atmospheric pCO_2 of 360 ppm, the air equilibrated seawater contains approximately 2 mM of DIC at 18 °C, 95 % of which is present as bicarbonate, with CO_2 representing only 10 µM. However, CO_2 levels below 10 µM and high pH values have been measured during bloom episodes in nature (Hinga 1992, Kukert and Riebesell 1998) and, on the other hand, atmospheric CO_2 levels near 600 ppm are predictible by the year 2100 if the current CO_2 emissions to atmosphere are maintained (Wigley *et al.* 1996). This increase would raise $CO_{2(aq)}$ in air-equilibrated seawater to levels around 25 µM. In our experiments, a wide range of $CO_{2(aq)}$ levels possible and predictable in natural ambients have been considered. The growth rate of *P. tricornutum* is saturated at high light and natural CO_2 levels since neither a CO_2 increase nor a DIC rise modify the measured growth rate. However, as was hypothesized by Burkhardt *et al.* (1999), growth at low light leads to a greater variation of C:N ratios to CO_2 limitation. Thus, the alteration observed in the biochemical composition and growth rates under light limited conditions show the relevance of energy requirement in the effectiveness of inorganic carbon uptake mechanisms. Use of bicarbonate, a competitive advantage in natural alkaline environments (Beardall 1991) could be restricted under low light conditions. If a future CO_2 rise in oceanic waters takes place, a concomitant pH decline would also occur and under these conditions the cell composition could be more dependent on the light availability rather than the DIC availability itself. In either case, this effect could vary significantly the carbon export ratio following bloom episodes of typical oceanic phytoplankton species.

Acknowledgements

This work was supported by a grant from Consejería de Educación y Ciencia de la Junta de Andalucía and by CICYT project AMB94-0684-CO2-O2 from Ministerio de Educación y Cultura (Spain). The authors thank the BIO II group from the Alfred Wegener Institute for providing us with *Phaeodactylum tricornutum*, clone CCAP 1052/1A. We also thank Drs J. J. Vergara, J. R. Andría, L. Prieto and I. E. Huertas for their kind revision and useful corrections of a draft of this manuscript. We are very grateful to Professor John Raven and another anonymous reviewer whose corrections considerably enhanced the manuscript.

Accepted 16 June 2002.

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