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Inorganic carbon availability and the growth of large marine diatoms

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ABSTRACT: To address the question of whether free CO_2 can be a growth-limiting nutrient in oceanic waters 3 large marine diatoms, *Stephanopyxis palmeriana* (Greville) Grunow, *Ditylum brightwellii* (T. West) Grunow in Van Heurck 1883, and *Coscinodiscus* sp., were grown in pH-drift experiments under batch culture conditions. The cultures were maintained under quiescent conditions without added buffer in the growth medium, allowing the pH to rise and the free CO_2 concentration to fall as growth proceeded. Growth rates were constant for sustained periods and only decreased as the pH rose to as high as 8.5–8.6 and the free CO_2 concentration fell to ~4 µmol l^{-1}. Such a low free CO_2 concentration is far below the expected half saturation coefficient for ribulose biphosphate carboxylase-oxygenase (RUBISCO) and suggests that these species were capable of utilizing HCO_3^- in some fashion so as not to allow free CO_2 to become growth-limiting. Additional experiments were conducted with these and other diatoms to demonstrate that turbulent mixing at high pH levels had no effect on inorganic carbon uptake or growth rate and that HCO_3^- uptake probably was occurring. Turbulent mixing should have enhanced inorganic carbon uptake by lowering diffusion gradients had these species been obligate users of free CO_2. These species, being the most susceptible to diffusion-controlled uptake of free CO_2 because of their long diffusion paths, represent the worst case scenario for free CO_2 limitation. Finally, by developing a chemical-biological model it was possible to show that, in order for the pH of marine waters to rise even several tenths above ambient levels, biomass concentrations must increase to levels that are never found, except possibly on occasion in estuarine or coastal waters. Thus it seems unlikely that free CO_2 limits phytoplankton growth in the oceans.

KEY WORDS: Inorganic carbon availability · pH levels · HCO_3^- uptake · Growth · Large marine diatoms

INTRODUCTION

Large marine diatoms, because they are able to respond effectively to episodic physical events, may be major contributors to new production in the open ocean (Goldman 1988, 1993). Moreover, they are the first trophic level in food chains that support the production of large fish (Ryther 1969, Michaels & Silver 1988, Moloney et al. 1991). Yet, we still do not fully understand how these organisms subsist in the open ocean and why and how they bloom on occasion. Although their nutritional requirements are typical of phytoplankton growing exponentially and their chemical composition is frequently found to be in Redfield proportions (Goldman et al. 1992), there still is uncertainty as to which of the commonly studied nutrients control their growth. Compared to the more sparingly available nutrients that might limit growth (nitrogen, phosphorus, silica, and, more recently observed, iron), total dissolved inorganic carbon ($C_t = CO_2 + HCO_3^- + CO_3^{2-}$) is present in the open ocean in great excess (~2 mmol l^{-1}) relative to phytoplankton needs. Hence, C_t historically has not been considered to be a limiting nutrient for growth even though carbon constitutes the largest fraction of phytoplankton biomass. The assumption underlying this conclusion is that all of C_t is a substrate for growth, either by the adequate supply of free CO_2 from the chemical equilibrium system $CO_2 \rightleftharpoons HCO_3^- \rightleftharpoons CO_3^{2-}$ or by direct uptake of HCO_3^-, or by both mechanisms. At the prevailing pH of seawater (~8.1 to 8.2) HCO_3^- is the dominant chemical species (~85 % of C_t) and free CO_2

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is present at a concentration of only 10 to 15 µmol l^{-1}. Even lower free CO_2 concentrations can occur at the cell surface when diffusion controls nutrient uptake (Gavis & Ferguson 1975, Wolf-Gladrow & Riebesell 1997). Thus if marine phytoplankton species were obligate users of free CO_2, it is conceivable that free CO_2 concentrations could fall to growth-rate-limiting levels (Gavis & Ferguson 1975, Riebesell et al. 1993). The recent claim that free CO_2 plays a major role in controlling marine primary productivity is based on this premise (Riebesell et al. 1993, Hein & Sand-Jensen 1997). If true, we would expect the effect to be greatest in large phytoplankton species that possess correspondingly thick diffusion boundary layers and hence long diffusion paths. As clearly shown by Koch (1971), the specific uptake rate of a solute controlled solely by diffusion is proportional to the reciprocal of the square of the cell diameter (see also Goldman 1984, Chisholm 1992, Kiørboe 1993). Hence, diffusion limitation becomes increasingly important as cell size increases.

A small rise in pH levels in natural waters resulting from phytoplankton growth can lead to a drastic reduction in the free CO_2 concentration. For example, when the pH of seawater rises to 8.3 the free CO_2 concentration drops to ~7 µmol l^{-1}; at pH 8.5 it is down to ~4 µmol l^{-1}. Thus if marine phytoplankton were obligate users of CO_2 we would expect to see large reductions in phytoplankton growth rates with even small increases in pH above ambient levels. Because of a great demand for inorganic carbon, large increases in culture pH, often to above 9, frequently occur in laboratory enrichment cultures (Goldman et al. 1982). As such, we would expect CO_2 limitation, if it exists at all, to be most prevalent in such an environment. In contrast, nutrients levels in the ocean generally are too low to support sufficient phytoplankton growth to cause even modest increases in pH, except in highly productive coastal and estuarine waters. Although oceanic pH data are scant, the pH of surface waters rarely exceeds ~8.2–8.3 (Clayton et al. 1995, Millero 1996). Thus through a simple 'pH-drift' experiment it should be possible to determine whether or not growth rates of marine phytoplankton are limited by free CO_2. Such an experiment would involve growing phytoplankton in enriched seawater without added buffers and under quiescent conditions. Then, by observing if changes in growth rate occur as the pH rises and the free CO_2 concentration falls, it should be possible to address definitively the question of obligate CO_2 use. Experiments of this type were performed in the current study with large marine diatoms since this phytoplankton group is most susceptible to diffusion control of CO_2 transport to the cell surface; hence, they would be among the first phototrophs to experience CO_2 limitation. Additionally, comparisons were made of cultures grown with and without mixing to determine the possible role of induced turbulence in enhancing growth rates by breaking down diffusion gradients of CO_2. And, finally, a simple chemical-biological model was developed to determine the growth conditions that would be required to raise seawater pH to levels commensurate with CO_2 limitation in obligate users of free CO_2. The results have bearing on the recent claim that free CO_2 limits growth of phytoplankton in seawater and on the resulting implications as to how global change might impact on marine primary productivity.

METHODS

Test species and enrichment medium. Four large diatoms, *Coscinodiscus* sp., *Ditylum brightwellii* (T. West) Grunow in Van Heurck (1883), *Odontella mobiliensis* (Bailey) Grunow, and *Thalassiosira weissfloggii* (Grun) Fryxell et Hasle, were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), while a fifth large diatom, *Stephanopyxis palmeriana* (Greville) Grunow, was collected by net tow from the Sargasso Sea during May 1989 and maintained in culture. The diatoms ranged in size from *T. weissfloggii* (12 × 20 µm) to *S. palmeriana* (100–120 × 50 µm) (see Table 1). The enrichment medium for all experiments was MET 44 (Schöne & Schöne 1982), consisting of 40 µM $NaNO_3$, 2.58 µM $NaHPO_4$, 35.7 µM Na_2SiO_3, 2.16 µM Na_2EDTA, 0.215 µM $FeSO_4$, 0.073 µM $MnCl_2$, and 0.5 µg l^{-1} each of vitamin B_{12}, biotin, and thiamine added to Sargasso seawater that had been filtered through glass fiber filters (Whatman GF/F). After the chemical additions, the seawater medium was pasteurized at 90°C for 2 h in polycarbonate bottles. After cooling the pH of the medium was ~8.1 (see below).

pH-drift experiments. Batch growth experiments were conducted in large Fernbach flasks containing 1.5 l of medium with 3 species, *Stephanopyxis palmeriana*, *Ditylum brighwellii*, and *Coscinodiscus* sp. The inoculum for each culture came from exponentially growing cells of stock cultures maintained on MET 44. Care was taken to ensure that starting cell concentrations were low enough (~1 to 2 cells ml^{-1} for *S. palmeriana* and *Coscinodiscus* sp. and <100 cells ml^{-1} for *D. brightwellii*) so as not to alter measurably the initial pH of the medium. Cultures were maintained in an environmental incubator (20°C and ~190 µEin m^{-2} s^{-1} continuous irradiance) and were vigorously mixed for ~10 s before sampling. Time-course sampling for cell counts, C_t, and pH commenced once visible growth was observed, usually within 2 to 3 d of inoculation. Samples were collected once daily in the early stages of each experiment, but more frequently when changes in pH occurred. Specific growth rates (μ in d^{-1})

were calculated by regression analysis as the slope of the linear portion of the curve of ln(cell number) versus time.

Turbulence experiments. To test the effect of induced turbulence on C_t uptake all 5 species first were grown under conditions identical to those in the pH-drift experiments. After 3 to 5 d of growth (depending on species) and after a noticeable rise in pH was observed, 1 h single-end-point or 24 h time-series $H^{14}CO_3^-$ labeling experiments were performed on mixed and unmixed samples. First, a small sample was taken for measurement of pH and C_t; after this a larger sample was taken, enriched with MET 44 nutrients, trace metals, and vitamins in the same concentrations used to grow the cultures, and then split into 2 equal portions. Approximately 1 µCi µmol C_t^{-1} $H^{14}CO_3^-$ was added to 1 portion and samples taken immediately for measurement of the specific activity of the C_t. Replicate 20 ml screw-capped vials were then filled, 1 with labeled culture for measurement of $H^{14}CO_3^-$ uptake and 1 with culture without label for measurement of pH at the end of the time point. Care was taken to avoid an air space in the vials. One set of vials was placed on a shaker table and the other set was kept unmixed. Both sets were positioned in front of a light bank and were exposed to about 190 µEin m^{-2} s^{-1}. The mixed vials were vigorously shaken at 250 rpm. Time-course measurements were made in experiments with 2 species (*Stephanopyxis palmeriana and Thalassiosira weissfloggii*) at 1, 4, 8, 12, and 24 h. Five vials containing labeled culture and 5 with culture without label were used for each treatment (mixed vs unmixed) so that a whole vial could be sacrificed at each time point. Experiments with the remaining 3 species (*Ditylum brightwellii, Coscinodiscus* sp. and *Odontalla mobviliensis*) were terminated after 1 h. Whole culture aliquots (1 ml) were taken at each sampling point and placed directly into scintillation vials containing 2 ml of methanol acidified with 5% glacial acetic acid. The vial contents were evaporated to dryness under an infra-red lamp, resuspended in 1 ml distilled water, followed by addition of 10 ml scintillation fluid (Handyfluor). Counts were then made on a Beckman LS 5000 TD liquid scintillation counter.

In a separate pH-drift experiment replicate cultures of *Ditylum brightwellii* were grown in large Fernbach flasks in the environmental chamber, 1 kept unmixed and 1 mixed on the shaker table at 175 rpm. A Teflon-coated stir bar was placed in the mixed culture to enhance turbulence. Time-course sampling for cell counts, pH and C_t was carried out as in the previous pH-drift experiments.

Chemical and biological measurements. Cell counts were made on samples preserved in Lugol's solution. Depending on the size of the species, counts were made with either a Spencer-Brightline hemocytometer, a Sedwick-Rafter slide or a 5 cm plastic Petri dish. Samples for pH and C_t measurements were obtained by vacuum filtering 25 ml of culture through GF/F filters (Whatman) under gentle vacuum (~25 mm Hg) to avoid degassing of free CO_2. The filtrate was collected in 20 ml glass vials attached directly under the exit stem of the filtration funnel. This allowed the vial to overflow with filtrate to minimize exposure to the air and eliminate an air space before the vial was removed from the filtration unit and sealed with a Teflon-lined screw cap. Separate vials were used for pH and C_t measurements. The C_t vials were either analyzed immediately or kept refrigerated until measurements were made, usually within several days of sampling. Measurements of pH were made immediately after sampling and filtration. Culture pH was measured on the NBS (National Bureau of Standards, Gaithersburg, MD, USA) scale with a Fisher Accumet combination probe and Model 825MP pH meter. Two-point standardization of the probe was performed with pH 7.0 and 10.0 NBS buffers (Fisher). Temperature was compensated for manually by recording buffer and sample temperatures as part of the buffer standardization protocol. The probe was immersed in a sample for 10 min before the pH was recorded. Although the meter displayed pH to 3 decimal places, pH data were rounded off to 2 places.

C_t was measured on a LiCor Model 6252 CO_2 Analyzer. Both temperature and barometric pressure were compensated for internally. A gas flow-through system was constructed consisting of a meter to maintain a continuous flow of carrier gas (ultra pure helium) at 120 ml min^{-1}, a glass chamber for sample sparging, an ice bath for collecting water vapor, a dessicant filter, and micro-particle filters. The analyzer was programmed to integrate and record the mass flow of CO_2. First, 0.1 ml of a 5% H_3PO_4 solution was injected through a rubber septum into the glass sparging chamber by syringe. Excess CO_2 in the acid was sparged out of the system before the integration sequence began. Next, a 1 ml sample was injected into the sparging chamber by syringe (Hamilton liquid-tight), C_t was converted to CO_2 in the acidified sample, and the integrating sequence was started. The efficiency of CO_2 extraction from C_t in the liquid phase virtually was 100%. The analyzer was calibrated with a range of standards from 0.25 to 2.5 mmol l^{-1} C_t made from mixtures of Na_2CO_3 and $NaHCO_3$. The resulting curve of meter reading versus C_t was fitted by non-linear regression analysis as a fifth-order polynomial.

Calculation of free CO_2 concentration. Free CO_2 concentration was determined from the equilibrium equations of the inorganic carbon system with pH and

C_t as the known variables (Stuum & Morgan 1981). Following the approach of Crawford & Harrison (1997), pH measurements made on the NBS scale (pH_{NBS}) were first converted to pH on the seawater scale (pH_{SEA}) using the relationship $pH_{SEA} = pH_{NBS} + \log f_H$ in which f_H is the activity coefficient for hydrogen ion. The magnitude of f_H is specific for each pH electrode and generally falls within a range of 0.70 to 0.85 (Mehrbach et al. 1973, Crawford & Harrison 1997). Although Crawford & Harrison (1997) obtained excellent agreement between measured and calculated values of free CO_2 when f_H was assumed equal to 0.85, in this study a more conservative value of 0.70 was assumed. With corrections for total fluoride and total sulfate (salinity = 35, temp = 20°C), pH_{SEA} was then converted to pH on the total scale (pH_{TOT}) and adjusted to mol (kg solution)$^{-1}$ (Millero 1995). The resulting change from pH_{NBS} to pH_{TOT} was −0.12 pH units. Hence, for the purposes of calculating free CO_2 from pH and C_t, all measured pH values were reduced by 0.12. Then free CO_2 was determined for each set of experimental pH and C_t values, using recommended values for the equilibrium coefficients of the inorganic carbon system (DOE 1994).

Modeling pH-biomass relationships. A simple chemical-biological model was constructed to determine the effect of phytoplankton growth and resulting increases in biomass on pH (Stuum & Morgan 1981). First, for a starting pH, alkalinity was determined as:

$$[ALK_I] = C_t(\alpha_1 + 2\alpha_2) + [OH^-]_I - [H^+]_I + [B(OH^-)_4]_I \quad (1)$$

in which $[ALK_I]$ is the initial alkalinity in eq l^{-1}, $[OH^-]_I$ and $[H^+]_I$ are, respectively, the hydroxyl and hydrogen ion concentrations in mol l^{-1} for the initial pH, $[B(OH^-)_4]_I$ is the initial borate concentration in mol l^{-1}, and $\alpha_1 + \alpha_2$ are the ionization fractions for HCO_3^- and CO_3^{2-}, respectively, as a function of pH. These coefficients are defined as:

$$\alpha_1 = (1 + [H^+]/K_1 + K_2/[H^+])^{-1} \quad (2)$$

and

$$\alpha_2 = ([H^+]^2/K_1K_2 + [H^+]/K_2 + 1)^{-1} \quad (3)$$

in which K_1 and K_2 are the equilibrium coefficients of the inorganic carbon system in mol l^{-1}. $[B(OH^-)_4]_I$ can be expressed as $B_TK_B/([H^+]_I + K_B)$ in which B_T is the total boric acid concentration in seawater and K_B is the dissociation coefficient for boric acid. Both B_T and K_B can be estimated for known salinity and temperature (Millero 1995). Changes in C_t (ΔC_t) (mol l^{-1} d^{-1}) due solely to phytoplankton growth were estimated by the relationship:

$$\Delta C_t = Q_C X_0(e^{\mu t} - 1) \quad (4)$$

in which Q_C is the carbon cell quota (mol cell^{-1}), X_0 is initial cell concentration (cells l^{-1}), μ is the specific growth rate (d^{-1}), and t is the duration of the growth period (d).

The final alkalinity (ALK_F) resulting from phytoplankton growth, while not changed by inorganic carbon uptake, is increased by both NO_3^- and HPO_4^{2-} uptake (Brewer & Goldman 1976, Stuum & Morgan 1981). Using a Redfield stoichiometry of $C_{106}N_{16}P_1$ for phytoplankton biomass, the change in alkalinity was calculated as:

$$ALK_F = ALK_I + (18/106)C_t \quad (5)$$

The factor 18/106 is derived from the fact that at the pH of seawater, HPO_4^{2-} is the dominant form of phosphate. Thus to maintain charge balance in the photosynthetic reaction, 18 mol of OH^- are produced for each mole of organic matter produced in the form $C_{106}N_{16}P_1$.

ALK_F can also be expressed as

$$ALK_F = (C_t - \Delta C_t)(\alpha_1 + 2\alpha_2) + [OH^-]_F - [H^+]_F + B_TK_B/([H^+]_F + K_B) \quad (6)$$

Eq. (6) was simplified by determining ALK_F from Eq. (5) and ΔC_t from Eq. (4) for known values of ALK_I (from Eq. 1), C_t, μ, Q_C, X_0, B_T, K_1, K_2, K_B, salinity and temperature and inserting them into the equation. Then pH_F (final pH) was determined as a function of time by varying H^+ in an iterative process until Eq. (6) was solved for each value of t. By assuming different growth scenarios, it was possible to gauge the impact of phytoplankton growth on seawater pH and to compare these results with growth conditions typically found in oceanic waters.

RESULTS

pH drift experiments

Growth rates of the 3 test species (*Stephanopyxis palmeriana*, *Coscinodiscus* sp., and *Ditylum brightwellii*) remained constant for extended periods in the static pH drift experiments while the pH rose and the free CO_2 concentration fell (Fig. 1). Levels of pH as high as 8.45 and 8.51, corresponding to minimum free CO_2 concentrations of 4.4 and 3.7 µmol l^{-1}, were observed, respectively, in the cultures of *S. palmeriana* (Fig. 1D) and *D. brightwellii* (Fig. 1F) before μ decreased. For *Coscinodiscus* sp., pH rose to a lower level than in the other 2 cultures mentioned above (8.31) and, free CO_2 decreased to 7.1 µmol l^{-1} before a decrease in μ was noted (Fig. 1E). There was no correlation between changes in pH or free CO_2 and μ or cell size: *D. brightwellii* displayed the highest μ (1.20 d^{-1}; Fig. 1C), followed by *Coscinodiscus* sp. (0.90 d^{-1}; Fig 1B) and *S. palmeriana* (0.50 d^{-1}; Fig. 1A) (Table 1).

Mixing had no impact on enhancing μ in a separate pH-drift experiment with *Ditylum brightweillii* (Fig. 2).

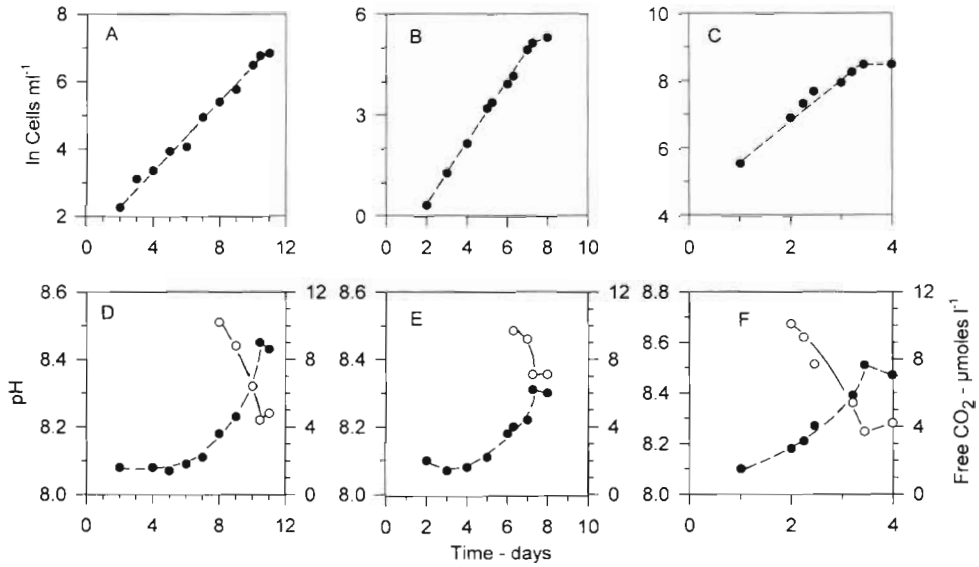

Fig. 1 Time course of growth, pH and free CO_2 changes during batch pH-drift experiments with large marine diatoms. (A,D) *Stephanopyxis palmeriana*; (B,E) Coscinodiscus sp.; (C,F) *Ditylum brightwellii*. (A–C) Changes in cell number; (D–F) changes in pH (•), and free CO_2 concentration (○). Curves in all figures (except linear portion of changes in cell number) were drawn by visual inspection to show trends only. Linear portion of changes in cell number was determined by regression analyses (same for Figs. 2 & 3)

After a few days of lag growth, growth rates were exponential and virtually identical in unmixed (1.91 d^{-1}; Fig. 2A) and mixed cultures (1.88 d^{-1}; Fig. 2B) (Table 1). However, the maximum pH was higher (8.54) and the minimum free CO_2 concentration lower (3.2 µmol l^{-1}) in the unmixed culture (Fig. 2C) compared to the mixed culture (maximum pH = 8.24, minimum free CO_2 concentration = 8.6 µmol l^{-1}; Fig. 2D) (Table 1). The lower pH in the mixed culture was attributed to enhanced gas exchange with the atmosphere.

Turbulence experiments

Initial growth rates of *Stephanopyxis palmeriana* and *Thalassiosira weissfloggii* before the 24 h $H^{14}CO_3$ uptake studies commenced in the presence and absence of mixing were, respectively, 0.81 and 1.14 d^{-1} (Fig. 3A,B). Rates of $H^{14}CO_3$ uptake of unmixed and mixed samples from both cultures were constant during the first 12 h of the 24 h incubation: 317 µmol C d^{-1} (both unmixed and mixed) for *S. palmeriana* (Figs. 3C & 4) and 812 (unmixed) versus 815 µmol C d^{-1} (mixed) for *T. weissfloggii* (Figs. 3D & 4). Based on linear regression analyses, r^2 was 0.98 to 0.99 for all curves up to 12 h incubation. Beyond 12 h incubation carbon uptake rates decreased for all samples. During the initial 12 h period free CO_2 concentrations dropped from 8.3 to 5.1 µmol l^{-1} and the pH rose from 8.26 to 8.41 for *S. palmeriana* (Fig. 3E) and from 2.2 to 0.4 µmol l^{-1} and 8.66 to 9.09 for *T. weissfloggii* (Fig. 3F) (Table 1).

Table 1. Summary of species size and experimental design and results. Turbulence experiment: pH-drift followed by 1 h or 24 h $H^{14}CO_3^-$ uptake experiment; $H^{14}CO_3^-$ uptake experiment initiated before maximum pH attained; values given in parentheses were taken at end of 12 h $H^{14}CO_3^-$ uptake experiment

Species	Dimensions (µm)	Expt	μ (d^{-1})	Max. pH	Min. CO_2 (µmol l^{-1})
Stephanopyxis palmeriana	50 × 100–120	pH-drift	0.50	8.45	4.4
		Turbulence	0.81	8.26 (8.41)	8.3 (5.1)
Coscinodiscus sp.	70–80 × 60–65	pH-drift	0.90	8.31	7.1
		Turbulence	0.74	8.21	9.3
Ditylum brightwellii	20–40 × 60–80	pH-drift	1.20	8.51	3.7
		Turbulence	1.56	8.42	5.0
		pH-drift (static)	1.91	8.54	3.2
		pH-drift (mixed)	1.88	8.24	8.6
Odontella mobiliensis	35–50 × 55–80	Turbulence	0.37	8.20	9.9
Thalassiosira weissfloggii	12 × 20	Turbulence	1.14	8.66 (9.09)	2.2 (0.4)

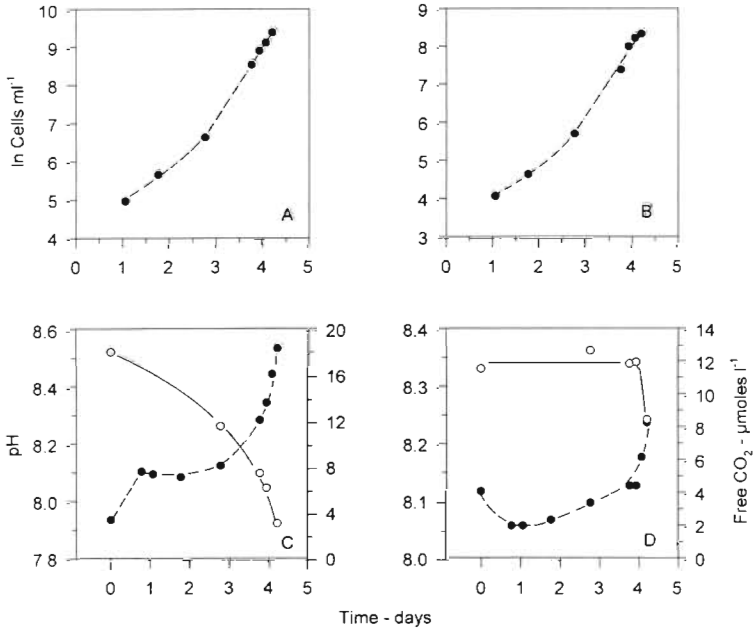

Fig. 2. *Ditylum brightwellii*. Time course of growth, pH and free CO_2 changes during batch pH-drift experiment. (A,C) static; (B,D) mixed. (A,B) Changes in cell number; (C,D) changes in pH (•) and free CO_2 concentration (o)

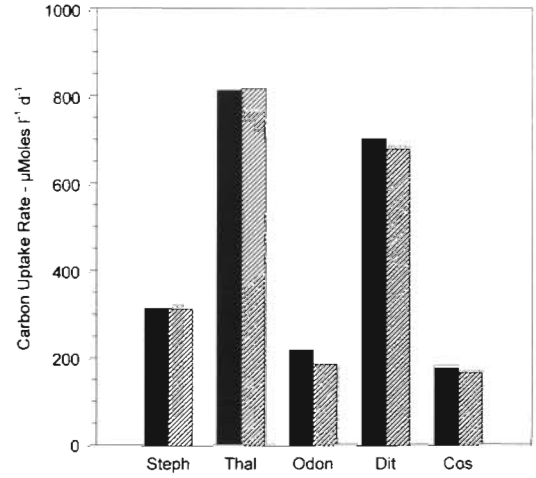

Fig. 4. Time series $H^{14}CO_3^-$ uptake of large marine diatoms: static (solid bar), mixed (shaded bar). Rate from slope of uptake curve from 24 h time series study shown in Fig. 3: *Stephanopyxis palmeriana* (Steph), *Thalassiosira weissfloggii* (Thal); 1 h rate from single-end-point measurement: *Odontella mobiliensis* (Odon), *Ditylum brightwellii* (Dit), *Coscinodiscus* sp. (Cos)

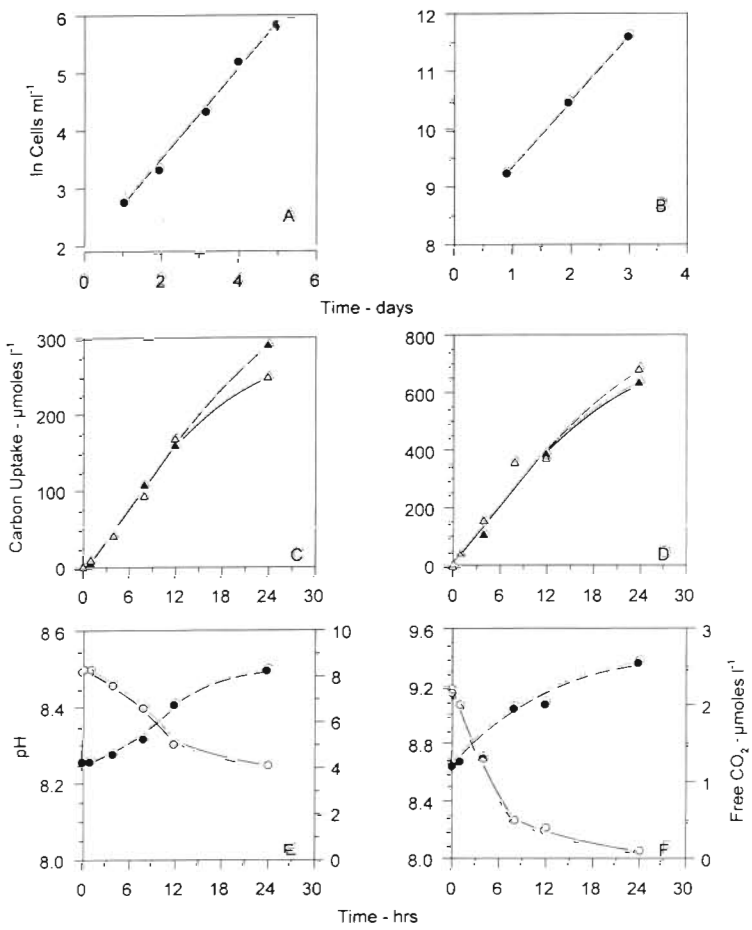

Fig. 3. Time course of growth during batch pH-drift phase and $H^{14}CO_3^-$ uptake, pH and free CO_2 concentration during short-term mixing phase with large marine diatoms. (A,C,E) *Stephanopyxis palmeriana*; (B,D,F) *Thalassiosira weissfloggii*. (A,B) Changes in cell number; (C,D) time series carbon ($H^{14}CO_3^-$) uptake: static (△), mixed (▲); (E,F) changes in pH (•) and free CO_2 concentration (o) (static and mixed were identical)

Growth rates of cultures used for the 1 h incubations were 0.74 d^{-1} for *Coscinodiscus* sp., 1.56 d^{-1} for *Ditylum brightwellii* and 0.37 d^{-1} for *Odontella mobiliensis* (Table 1). As during the 12 h incubations, there was virtually no difference in carbon uptake rates for unmixed and mixed samples for the 3 species (Fig. 4). Also, both free CO_2 concentration and pH remained unchanged during the short 1 h incubations for all 3 species, whether mixed or unmixed. Free CO_2 concentration and pH were, respectively, 9.3 µmol l^{-1} and 8.21 for *Coscinodiscus* sp., 5.0 µmol l^{-1} and 8.42 for *D. brightwellii*, and 9.9 µmol l^{-1} and 8.20 for *O. mobiliensis* (Table 1).

Effect of growth on pH

Two growth scenarios similar to those of the current experiments were modeled according to Eqs. (4) to (6), one with *Stephanopyxis palmeriana* and the other with *Thalassiosira weisfloggii*. For *S. palmeriana* the

effect of growth on pH_F (final pH) was examined for 2 initial values of pH (pH_I = 8.1, 8.2), an initial cell number X_0 of 1600 cells l^{-1} and a specific growth rate μ of 0.8 d^{-1} (from Table 1). Values of 20°C for temperature, 35 for salinity, and 2.3 mmol l^{-1} for C_t were assumed and Q_c (5000 pg $cell^{-1}$) and cellular chlorophyll *a* (chl *a*) (120 pg $cell^{-1}$) for this species were obtained from a previous study (Goldman et al. 1992). From the resulting curves of increasing biomass levels (represented by chl *a*) (Fig. 5A) and pH_F (Fig. 5B) and decreasing free CO_2 concentration (Fig. 5C) over time, it is obvious that only when the chl *a* concentration rises above ~10 µg l^{-1} are there appreciable changes in pH and free CO_2 concentration from the starting values. For example, production of 10 µg chl *a* l^{-1} leads to a pH increase of only <0.1 pH units for both values of pH_I and a reduction in free CO_2 concentrations to ~10.4 µmol l^{-1} for pH_I of 8.1 and 8.2 µmol l^{-1} for pH_I of 8.2. In contrast, production of 50 µg chl *a* l^{-1} results in an increase of ~0.35 pH units for both pH_I values so that pH_F increases to ~8.45 or ~8.55 and the free CO_2 concentration decreases to ~5 or ~4 µmol l^{-1} when pH_I is 8.1 or 8.2.

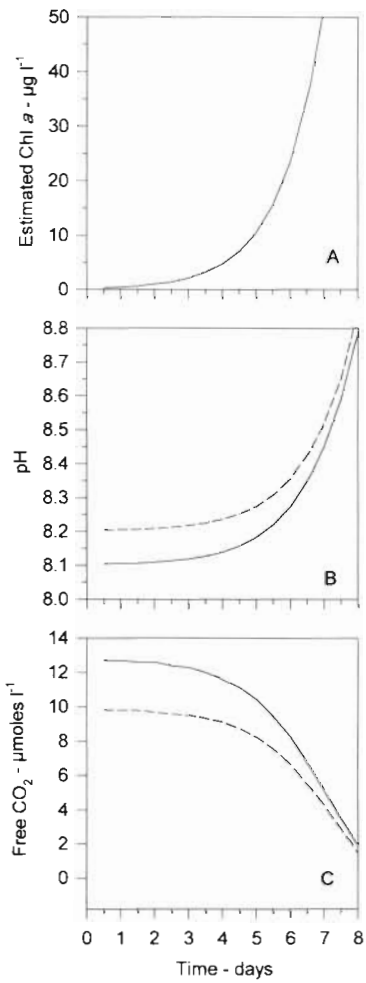

Fig. 5. *Stephanopyxis palmeriana*. Model scenario of effects of growth for different starting pH values (pH_I) on (A) chl *a*, (B) pH_F and (C) free CO_2 concentration. (B,C) ——: pH_I = 8.1; – – –: pH_I = 8.2. See text for growth conditions

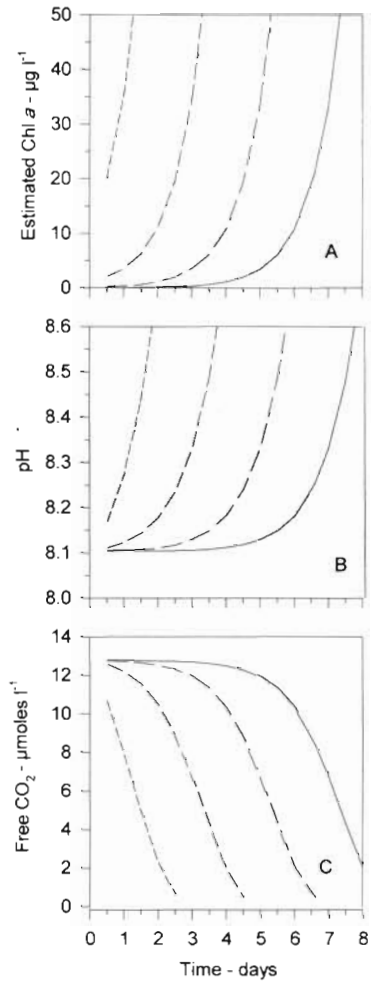

Fig. 6. *Thalassiosira weissfloggii*. Model scenario of effects of growth for different starting cell concentrations (X_0) on (A) chl *a*, (B) pH_F and (C) free CO_2 concentration. – – –: $X_0 = 2 \times 10^3$ cells ml^{-1}; — — —: $X_0 = 2 \times 10^4$ cells ml^{-1}; — — · — · $X_0 = 2 \times 10^5$ cells ml^{-1}; ——: $X_0 = 2 \times 10^6$ cells ml^{-1} See text for growth conditions

Growth conditions for the second scenario involving *Thalassiosira weisfloggii* included μ = 1.14 d^{-1} (from Table 1), pH_I = 8.1, and the same temperature, salinity, and C_t as in the previous scenario. For this case, however, the aim was to determine the impact on pH_F of varying X_0, in this case over a span of 3 orders of magnitude from 2×10^3 to 2×10^6 cells l^{-1}. Estimates of Q_c (226 pg $cell^{-1}$) and cellular chl *a* (5.65 pg $cell^{-1}$) for this species also came from a previous study (Goldman & Glibert 1982). The results obtained were strikingly similar to those involving *Stephanopyxis palmeriana*.

Only when chl *a* levels exceeded ~10 µg l^{-1} (Fig. 6A) were there appreciable changes in pH (Fig. 6B) and free CO_2 concentration (Fig. 6C). The starting cell number had little effect on these results, only the time it took to achieve these changes. At chl *a* levels of 10 µg l^{-1} the increase in pH was <0.1 pH units, whereas at 50 µg l^{-1} the increase was ~0.35 pH units (pH_F ~8.45). Correspondingly, the free CO_2 concentrations fell to about 11 and 6 µmol l^{-1} at these chl *a* levels.

DISCUSSION

Free CO_2 requirements for growth

In the context of trying to interpret the data from the pH-drift and turbulence experiments and to determine the conditions that might favor limitation by free CO_2 of phytoplankton growth rates in seawater it is necessary to consider 2 essential points. First, in C_3 plants, which include marine phytoplankton, free CO_2 is required internally in the initial reaction of the photosynthetic process. The enzyme ribulose biphosphate carboxylase-oxygenase (RUBISCO), which catalyzes this reaction, has a rather high half saturation coefficient (K_S) for CO_2, about 25 to 40 µmol l^{-1} for green algae and diatoms and 100 to 120 µmol l^{-1} for cyanobacteria (Jordan & Ogren 1981, Read & Tabita 1994). And, second, it is well documented that K_S values for free CO_2 uptake by numerous marine phytoplankton species typically are below 1 to 2 µmol l^{-1} (Raven & Johnson 1991).

Thus in order to reconcile the great difference in the magnitudes of the 2 K_S values and to avoid CO_2 limitation of photosynthesis there must be some mechanism for elevating the free CO_2 concentration at the site where RUBSICO is located. Indeed, from both the plant physiology and marine biology literature, there is now substantial evidence that many different species, including marine diatoms, have the ability to concentrate free CO_2 internally (Lucas & Berry 1985, Burns & Beardall 1987, Munoz & Merrett 1989, Badger & Price 1992, Korb et al. 1997, Nimer et al. 1997, Tortell et al. 1997). The process is believed to occur either by the facilitated conversion of HCO_3^- to CO_2 externally, followed by transport of CO_2 into the cell, or by the active uptake of HCO_3^- into the cell, followed by conversion internally to CO_2 (Badger & Price 1992). The enzyme carbonic anhydrase plays a central role in catalyzing the conversion of HCO_3^- to CO_2 in both processes and is produced by the cell when the external free CO_2 concentration is reduced below a critical level (Badger & Price 1994). In comparing the low external free CO_2 concentration in seawater at pH 8.1 to 8.2 with the high K_S for CO_2 of RUBISCO, it is easy to understand why the CO_2 concentrating step is vital to prevent inorganic carbon limitation in marine phytoplankton, particularly in large species.

It is intuitively obvious that some mechanism for active uptake of inorganic carbon was occurring in the pH-drift experiments and that free CO_2 was not limiting growth of the 4 large diatoms. Otherwise, it would have been impossible to sustain constant growth rates as the free CO_2 concentration dropped to levels 3 to 6 times lower than typical seawater values and 6 to 20 times lower than the K_S for RUBISCO. Had these diatoms been obligate users of free CO_2, then a reduction in μ should have occurred over this range of free CO_2 concentrations. Given the large size of the 4 diatoms in the pH-drift experiments (Table 1) and the associated long diffusion paths, even lower free CO_2 concentrations probably occurred at the sites of uptake. Such a diffusion constraint, had it existed, most likely would have been most severe in *Stephanopyxis palmeriana*, the largest of the test species. Individual cells of *S. palmeriana*, which are very large (50 × 100 to 120 µm), grow in very long intertwined and overlapping chains (10s to 100s of cells in length). The chains form loosely bound aggregates that are visible to the naked eye (Goldman 1993) and in the absence of liquid shear possess longer diffusion paths than single cells (Pahlow et al. 1997).

The fact that strong mixing had no effect on either growth (Fig. 2), or ^{14}C uptake (Figs. 3 & 4) adds further support to the arguments that free CO_2 was not controlling growth and that facilitated HCO_3^- uptake most likely was occurring. It is well established that the degree of turbulence that typically occurs in the oceans only benefits very large phytoplankton cells (>50 µm) in breaking down diffusion barriers at the cell surface (Lazier & Mann 1989, Karp-Boss et al. 1996). As pointed out by Karp-Boss et al. (1996), it is virtually impossible to simulate in the laboratory the form of turbulence present in the ocean, even with sophisticated 'Couette' type devices that allow measurement of shear rates. Thus to provide a simple qualitative demonstration of whether or not phytoplankton growth rates could be enhanced by mixing, intense irregular mixing provided by a shaker table operated at maximum speeds was used. Mixing experiments of this type have been used successfully in the past by Pasciak & Gavis (1975) to demonstrate the effects of diffusion in controlling nutrient uptake rates of *Ditylum brightwellii*, the same species as used in the current study. At speeds of 175 rpm for the small glass vials and 250 rpm for the larger Fernbach vessel, the shaker table was operated at close to its maximum attainable speeds. While it is impossible to quantify the degree of turbulence experienced in these vessels, it should have been greater than even the most intense levels found

in the ocean, which are on the order of ~10^{-2}–10^{-3} cm^2 s^{-3} (Gargett 1989). Thus at least the 2 large species in this experiment (*Stephanopyxis palmeriana* and *D. brightwellii*), which were capable of drawing down the free CO_2 concentration in the bulk fluid to ~5 µmol l^{-1} when mixed (Table 1), should have experienced an increase in uptake of CO_2 if they were obligate users of free CO_2.

pH and nutrient enrichment

Given that the C_t concentration is remarkably constant throughout the world's oceans at ~2.0–2.3 mmol l^{-1}, it is evident that pH is the major determinant of the free CO_2 concentration in seawater. And, in turn, the magnitude of nutrient enrichment determines the level of phytoplankton biomass and, concomitantly, the resulting pH and free CO_2 concentration of a growing cell population. Hence, because enrichment media for growing cultures in the laboratory generally contain nutrient concentrations far in excess of those found in even the most productive waters, it is expected that the final biomass and resulting pH of a growing laboratory culture will be considerably greater than that found in natural waters, even under bloom conditions. For example, enrichment media such as MET 44 can support levels of phytoplankton biomass that far exceed those found even in the most productive coastal upwelling and estuarine waters. Although chl *a* was not measured in the current study, multiplying the cell concentration of 664 cells ml^{-1} attained before μ decreased for *Stephanopyxis palmeriana* (Fig. 1A) by the cellular chl *a* quota of 120 pg $cell^{-1}$ for this species (from Goldman et al. 1992), leads to a chl *a* production of ~80 µg l^{-1}. This chl *a* concentration is almost identical to what was attained for *S. palmeriana* and other large diatoms grown on MET 44 medium in experiments similar to the current ones (Goldman et al. 1992). It exceeds the chl *a* concentration typically found in highly productive upwelling and estuarine waters (up to ~10–25 µg l^{-1}) by up to an order of magnitude and in oceanic waters (~0.05–0.1 µg l^{-1}) by several orders of magnitude (Chavez et al. 1996, Cloern 1996, Wells pers. comm.). The results from the modeling exercises (Figs. 5 & 6), in which at least 50 µg l^{-1} chl *a* was required before the pH and free CO_2 concentration of seawater approached levels where reductions in growth rate might occur, highlight this point. Simply, such biomass levels, while common in enriched laboratory cultures, are never found in the ocean.

If the responses of the large diatoms in the current experiments are representative of indigenous species then it is unlikely that free CO_2 concentration has much, if any, influence on growth rates of marine phytoplankton, since the large diatoms, because of their long diffusion paths, represent the worst case scenario. This conclusion leads to the important point that in trying to use results from laboratory experiments to infer the conditions under which free CO_2 might limit marine phytoplankton growth in natural waters it is necessary to compare not only biomass levels of the 2 systems, but the pH and free CO_2 concentration as well. While good pH data are lacking for oceanic waters, there are an abundance of high quality free CO_2 data, showing that the pCO_2 concentration in the world's oceans rarely falls below 200 µatm (equivalent to 6.5 µmol l^{-1} free CO_2 at 20°C) and most often is between 300 and 400 µatm (9.7 to 12.9 µmol l^{-1} free CO_2 at 20°C) (Landrum et al. 1994, Robertson et al. 1994, Bates et al. 1996, Cooper et al. 1998, Wanninkhof & Feely 1998). Much of the variability is related to physical phenomena such as temperature, wind speed, and mixing, but in some productive locales, such as the northeast Atlantic where coccolithophore blooms are common and in the Bering Sea during the spring bloom, decreases in pCO_2 down to 150 to 200 µatm have been measured (Codspoti et al. 1986, Cooper et al. 1994, Robertson et al. 1994). However, even the lowest pCO_2 levels measured in such regions are above the CO_2 levels associated with reductions in μ of the large diatoms from the current study, which were about 2 to 4 µmol l^{-1} (equivalent to 60 to 120 µatm at 20°C).

ECOLOGICAL PERSPECTIVE

It is important to note that the levels of pH and free CO_2 concentration at which μ began to decrease in the current experiments were not necessarily the result of free CO_2 limitation. Although such a conclusion has been inferred previously by Riebesell et al. (1993) and Chen & Durbin (1994) in experiments where pH and free CO_2 concentration were co-varied, it is virtually impossible to draw any conclusions about free CO_2 effects on μ unless pH is held constant. Both adverse physiological responses and reduced availability of sparingly soluble and essential macro- and micronutrients at higher pH could impact on growth. What is most important, however, is that, in order to observe a reduction in growth rate in the current experiments, a pH level of about 8.5 to 8.7 was necessary for the large diatoms. Similar results were obtained by Riebesell et al. (1993) and Chen & Durbin (1994) in experiments involving other marine diatom species, although they did not conclude, as in this study, that such conditions are mainly restricted to enrichment cultures. As stated previously, such extreme pH levels and correspond-

ingly low CO_2 concentrations never occur in the ocean, except perhaps during exceptional blooms in isolated coastal and estuarine environments.

In conclusion, the seemingly widespread ability of marine phytoplankton to utilize HCO_3^-, in principle, allows full utilization of the reservoir of inorganic carbon in oceanic waters. Thus it is necessary to look to other more sparingly available nutrients as controlling factors of marine primary productivity. The recent interest in iron as a limiting nutrient in some regions of the world's ocean has highlighted the possibility that low levels of other trace metals may also play a role in regulating productivity. For example, zinc (a co-factor in carbonic anhydrase, the enzyme necessary for HCO_3^- uptake), like iron, is found in potentially limiting concentrations in some regions of the ocean (Bruland 1989). Morel et al. (1994) have shown in laboratory studies that limiting levels of zinc indirectly influence inorganic carbon uptake by the diatom *Thalassiosira weissfloggii* through effects on carbonic anhydrase activity. A similar effect was found in other, but not all, marine phytoplankton species by Lee & Morel (1995). In addition, it was found that cadmium and cobalt can in part substitute for zinc (Price & Morel 1990, Lee & Morel 1995, Sunda & Huntsman 1995).

In the current study the only trace metals purposely added to Sargasso seawater were iron and manganese. However, neither clean techniques nor purified reagents were used in the preparation of the medium and inadvertent contamination by zinc likely occurred, thereby negating any possible influence of this metal on the activity of carbonic anhydrase and concomitantly on growth rate as a function of increasing pH. Nonetheless, it is virtually impossible to draw any conclusions about how trace metals might have impacted on phytoplankton growth in the present study, given the enormous complexities of trace metal interaction and regulation of phytoplankton growth (Hudson & Morel 1993). From an ecological standpoint it would seem that oceanic phytoplankton have evolved the ability to utilize HCO_3^- in some fashion as a means of avoiding limitation by free CO_2. Whether a similar evolutionary adaptation exists for coping with low levels of trace metals in oceanic waters, and how it might impact on inorganic carbon acquisition by marine phytoplankton, remains to be determined. While such questions were beyond the scope of the present study, they are worthy of considerable future research.

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