

## Inorganic carbon acquisition in coastal Pacific phytoplankton communities

*Philippe D. Tortell*

Department of Ecology and Evolutionary Biology, Princeton University, Princeton, New Jersey 08544

*Greg H. Rau*

Institute of Marine Sciences, University of California, Santa Cruz, Santa Cruz, California 95064

*François M. M. Morel*

Department of Geosciences, Princeton University, Princeton, New Jersey 08544

### *Abstract*

Despite significant advances in the understanding of carbon acquisition in eukaryotic algae and cyanobacteria, very little information is available on the mechanisms of C uptake in natural phytoplankton communities or the effects of CO<sub>2</sub> variations on marine primary productivity. In this article, we present the results of a 3-yr study of C acquisition in coastal Pacific phytoplankton populations and their responses to experimental CO<sub>2</sub> manipulations. Diatom-dominated phytoplankton assemblages collected without incubation showed photosynthetic characteristics indicative of a carbon concentrating mechanism. Cells possessed a high affinity for external inorganic C (apparent  $K_m \sim 1 \mu\text{M CO}_2$ ) and accumulated internal inorganic C pools that were  $\sim 3$ – $5.5$ -fold higher than those in the external medium. Evidence of in situ carbonic anhydrase expression was found in some of the phytoplankton populations we examined, and inhibitor experiments showed that this enzyme was essential for C fixation by cells. The presence of carbon concentrating mechanisms enabled the phytoplankton to maintain rapid growth rates over a wide range of CO<sub>2</sub> concentrations (3–32  $\mu\text{M}$ ). In five of six long-term ( $\sim 2$ – $5$ -d) CO<sub>2</sub> manipulation experiments, no difference in growth rates could be detected across treatments. However, a significant decrease in growth rate (30%) was observed in one experiment at the lowest CO<sub>2</sub> level tested (3  $\mu\text{M}$ ). Although phytoplankton growth rates were generally unaffected by the CO<sub>2</sub> manipulations, significant CO<sub>2</sub>-dependent changes occurred in the cellular biochemistry and physiology of two assemblages that were examined. Cells grown at low CO<sub>2</sub> showed higher short-term rates of C uptake (indicative of transport system up-regulation), as well as enhanced expression of Rubisco and carbonic anhydrase. In one of these two incubation experiments, lower C:N and carbohydrate:protein ratios were observed at low CO<sub>2</sub>. Phytoplankton from both incubations showed low C isotope discrimination relative to the <sup>13</sup>C/<sup>12</sup>C of the available CO<sub>2</sub>. Photosynthetic fractionation factors ( $\epsilon_p$ ) ranged from  $\sim 3.5\%$  to 7.5% and were independent of both cellular growth rates and aqueous CO<sub>2</sub> concentrations. Our data indicate that nutrient-replete, rapidly growing coastal phytoplankton can use carbon concentrating mechanisms and respond physiologically and biochemically to changing dissolved CO<sub>2</sub> concentrations. Future field studies should examine the effects of CO<sub>2</sub> on the growth of nutrient-limited phytoplankton and assess the potential long-term ecological shifts that may result from CO<sub>2</sub> variations.

Over the past 2 decades, laboratory studies of inorganic C acquisition in phytoplankton have led to the discovery and partial characterization of carbon concentrating mechanisms (CCMs) in a variety of eukaryotic algae and cyanobacteria (Raven 1997; Badger et al. 1998; Kaplan and Reinhold

1999). Species that use CCMs accumulate high intracellular CO<sub>2</sub> concentrations in order to increase the efficiency of net C fixation by Rubisco and repress the enzyme's energetically wasteful photorespiratory activity (Canvin 1990). Carbon concentrating mechanisms involve the active uptake of HCO<sub>3</sub><sup>-</sup> and/or CO<sub>2</sub> from the environment and generally require the enzyme carbonic anhydrase (a Zn metalloenzyme that sometimes contains Co or Cd in the active site; Coleman 1998; Lane and Morel 2000a, 2000b) to catalyze the interconversion of these inorganic carbon species (Sültemeyer 1998). For the majority of phytoplankton thus far examined (both marine and freshwater), some form of CCM appears to be essential for maximum growth in air-equilibrated waters in which the concentration of aqueous CO<sub>2</sub> ( $\sim 10 \mu\text{M}$ ) falls well below the half-saturation constant of Rubisco ( $K_m$  range  $\sim 20$ – $200 \mu\text{M}$ ; see Badger et al. 1998). By regulating the activity of the CCM through changes in cellular biochemistry, phytoplankton are able to maintain rapid growth rates over a wide range of CO<sub>2</sub> concentrations. The response of cells to variations in aqueous CO<sub>2</sub> may, however, depend upon the availability of resources such as light, nitrogen, and

### *Acknowledgments*

We wish to thank Ken Bruland, Mark Wells, and C. S. Wong for generously providing space and research support on vessels and Geoff Smith and Ken Johnson for designing and maintaining trace metal-clean sampling systems. Dianne O'Brien assisted with experiments on the 1998 *Pt. Sur* cruise, and the crews and scientists of the R/V *Pt. Sur* and CCGS *JP Tully* provided essential logistical support on all trips. We also thank Klaus Keller for stimulating discussions throughout the preparation of the manuscript and for significant assistance with the analysis and interpretation of C isotope data. Laboratory advice and assistance were provided by Todd Lane and Irene Schaperdorth. This work was supported by the NSF and by FCAR and NSERC graduate fellowships to P.D.T. G.H.R. acknowledges the laboratory assistance of C. Low and support from the NSF (OCE 9618626), Seagrant (R/CZ 134), and Lawrence Livermore National Laboratory (B505001).

trace metals, which are required for the acquisition of inorganic C (Raven and Johnston 1991).

To date, nearly all studies of C acquisition in phytoplankton have focused on a small number of (mostly freshwater) model organisms grown in monospecific, nutrient-replete laboratory cultures. Few studies have examined the pathways of C uptake and assimilation in natural phytoplankton populations or the potential for C limitation of primary production in situ. Most field work on C limitation in aquatic systems has been done in alkaline freshwaters, in which photosynthetic activity can lead to significant CO<sub>2</sub> depletion. Early studies (reviewed by Talling 1976) suggested that low CO<sub>2</sub> availability could indeed constrain primary productivity in such environments. More recent physiological work has demonstrated that CCMs are important for the growth of phytoplankton in some lakes (Berman-Frank et al. 1994, 1998).

Since the pioneering work of Morris and colleagues on the pathways of inorganic C assimilation and fixation in natural marine phytoplankton assemblages (see Morris 1980), few oceanographic field studies of inorganic C acquisition have been undertaken. Given the abundance of inorganic C in seawater relative to other nutrients (nitrate, phosphate, silicate, and trace metals), it has typically been assumed that C availability does not limit photosynthesis in the oceans (e.g., Lalli and Parsons 1994). However, this notion has recently been challenged by a series of laboratory results demonstrating that the growth of large marine phytoplankton (which contribute disproportionately to biological C export from surface waters) can potentially be limited by CO<sub>2</sub> diffusion (Riebesell et al. 1993) and that HCO<sub>3</sub><sup>-</sup> utilization by these organisms may be inhibited by low oceanic concentrations of certain trace metals (Morel et al. 1994).

Inorganic C acquisition by marine phytoplankton has recently attracted growing interest and debate among biological oceanographers and marine biogeochemists. Interpretations of sedimentary C isotope records—and their use to reconstruct past variations in oceanic CO<sub>2</sub> concentrations—have generally been based on models that assume diffusive CO<sub>2</sub> uptake by phytoplankton and predict a linear relationship between isotope fractionation and the ratio of growth rate to CO<sub>2</sub> concentration (Jasper et al. 1994). An increasing number of laboratory and field studies have suggested, however, that the relationship between CO<sub>2</sub> concentrations and C isotope discrimination may be complicated by active C transport (Laws et al. 1997; Pancost et al. 1997, 1999; Burkhardt et al. 1999b; Rau et al. in press). The mechanisms of C acquisition employed by phytoplankton in situ should also affect their responses to changing CO<sub>2</sub> levels and the potential for CO<sub>2</sub> limitation of oceanic primary production. Although it is now firmly established that marine phytoplankton—diatoms, in particular—play an important role in oceanic CO<sub>2</sub> uptake through the sinking flux of organic C to the deep sea (Sarmiento and LeQuéré 1996), the effects of CO<sub>2</sub> variations (both natural and anthropogenic) on the efficiency of the biological carbon pump remain unknown.

Recently, several studies examining the short-term (<<1 d) photosynthetic responses of marine phytoplankton populations to CO<sub>2</sub> manipulations have reported some evidence for enhanced carbon fixation rates at elevated CO<sub>2</sub> (decreased

pH; Chen and Durbin 1994; Hein and Sand-Jensen 1997). These short-term results, however, may not necessarily reflect the steady-state responses of phytoplankton to CO<sub>2</sub> variations, and they provide no information on the physiological mechanisms of C acquisition employed by indigenous species. In an earlier publication (Tortell et al. 1997), we presented evidence that natural assemblages of coastal diatoms possessed the capacity for active C uptake and were able to maintain constant growth rates (in a 3-d incubation experiment) over a range of CO<sub>2</sub> concentrations (~3–25 μM) encompassing those typically encountered in the oceans. In this paper, we extend these initial results, with data from a 3-yr field study of C acquisition in coastal Pacific Ocean phytoplankton communities. We present evidence that phytoplankton operate active C transport mechanisms in situ and examine the effects of CO<sub>2</sub> manipulations on cellular growth rates, inorganic C acquisition, biochemical composition, and stable C isotope fractionation. The implications of our results to future studies of C limitation in the oceans are discussed.

## Materials and methods

*Field sites*—Field experiments were conducted in the spring and summer (May–July) of 1996–1998 in coastal waters of the northeast Pacific Ocean. Sampling sites were located along the west coast of North America (mostly < 20 km) from California to British Columbia. This coastal region is characterized by seasonally intense upwelling of nutrient-rich deep waters that support high primary productivity dominated by large (>30 μm) solitary and chain-forming diatoms (Lalli and Parsons 1994). Significant spatial and temporal variability exists in nutrient supply (macronutrients and trace metals) and phytoplankton biomass in these waters. During opportunistic sampling from hydrocasts on the cruises, we measured mixed-layer chlorophyll *a* concentrations ranging from <1 to >30 μg L<sup>-1</sup>, NO<sub>3</sub><sup>-</sup> ranging between <<1 and >30 μM, and CO<sub>2</sub> ranging between 6 and 25 μM. Recent work indicates that phytoplankton growth in certain regions of the coast is limited by trace metal (Fe) availability (Hutchins and Bruland 1998). All field work was conducted aboard the R/V *Pt. Sur* and CCGS *JP Tully*.

*In situ sampling and photosynthesis experiments*—Phytoplankton were collected from the surface mixed layer by passing water from Niskin bottles (typically 2–5 L) through 3-μm in-line polycarbonate filters or by filtering concentrated samples from plankton net tows. For shipboard photosynthesis measurements, algae were immediately resuspended off filters into 1–2 ml of a carbon-free seawater buffer. To prepare this buffer, 0.2 μm filtered seawater with 10 mM added Bicine was acidified to pH 4.0, sparged with N<sub>2</sub> for ~1 h, and titrated back to pH 8.2 with NaOH pellets. Cell suspensions (~0.5–2 μg Chl *a* ml<sup>-1</sup>) were placed in an illuminated (~600 μmol photons m<sup>-2</sup> s<sup>-1</sup>), water-cooled O<sub>2</sub> electrode (Hansatech) and allowed to deplete any residual inorganic C in the system (extracellular and internal pools). Inorganic C depletion was assumed to be complete when rates of photosynthesis (O<sub>2</sub> production) were balanced by respiration (O<sub>2</sub> consumption) and no net change in O<sub>2</sub> concentrations could be measured in the electrode.

Rates of short-term  $^{14}\text{C}$  incorporation into cellular organic and inorganic pools were measured by use of the silicone oil centrifugation technique (Badger et al. 1980). This method has been widely used in laboratory studies of C acquisition in phytoplankton. Briefly, 150–200  $\mu\text{l}$  aliquots of C-depleted cell suspensions (stored in tightly capped,  $\text{N}_2$  sparged vials) were added to microcentrifuge tubes that contained 85  $\mu\text{l}$  of 2.5N NaOH in 10% methanol (bottom layer) and 130  $\mu\text{l}$  silicone oil (middle layer). Individual tubes were illuminated with a slide projector ( $\sim 600 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) and varying amounts of  $\text{H}^{14}\text{CO}_3^-$  solution ( $\sim 50 \text{ mCi mmol}^{-1}$ ; pH  $\sim 8.2$ ) were injected with a pipette. Immediately after the  $^{14}\text{C}$  additions, cell suspensions were mixed by repeatedly withdrawing and dispensing solution from immersed pipette tips. After a brief period of incubation (5–60 s), tubes were rapidly transferred into a microcentrifuge and spun at  $14,000 \times g$  to separate cells from the  $^{14}\text{C}$  solution by drawing them through the silicone oil into the NaOH/methanol solution. This separation required  $< 5$  s. Tubes were immediately frozen in liquid nitrogen, and cell pellets were cut off and resuspended in scintillation vials containing 1 ml of 0.1N NaOH. Half of the resuspended pellet solutions (i.e., 500  $\mu\text{l}$ ) was transferred into fresh vials, acidified with 500  $\mu\text{l}$  of 0.5 N HCl, and left to degas  $^{14}\text{CO}_2$  for at least 12 h. The amount of total (inorganic + organic) and acid-stable (organic)  $^{14}\text{C}$  in cell pellets was determined by liquid scintillation counting and the inorganic C fraction determined by difference.

Intracellular C concentrations were calculated by normalizing  $^{14}\text{C}$  activity in cell pellets to biovolume, which was measured by incubating samples for 1–2 min with  $^3\text{H}_2\text{O}$  and  $^{14}\text{C}$ -sorbitol and centrifuging as described above. These labeled compounds provide an estimate of total biovolume ( $^3\text{H}_2\text{O}$ ) corrected for  $^{14}\text{C}$  contamination in extracellular  $\text{H}_2\text{O}$  ( $^{14}\text{C}$ -sorbitol) (Badger et al. 1980). Biovolumes in concentrated cell pellets used in the experiments averaged 0.21 ( $\pm 0.02$ )  $\mu\text{l}$ , whereas extracellular water taken down with cells (measured with  $^{14}\text{C}$  sorbitol) averaged  $\sim 0.11$  ( $\pm 0.04$ )  $\mu\text{l}$  for all samples. This ratio of cellular to extracellular water volume is similar to that reported previously in work with laboratory diatom cultures (Mitchell and Beardall 1996; Johnston and Raven 1997). In all experiments, phytoplankton  $^{14}\text{C}$  uptake represented only a small fraction ( $< 1\%$ ) of the total added  $^{14}\text{C}$  label, and at most 15% of the inorganic C in cell pellets could be accounted for by contamination from extracellular  $^{14}\text{C}$ -labelled dissolved inorganic carbon (DIC) carried through the silicone oil layer with cells.

In time-course experiments, changes in the cellular organic and inorganic C pools were followed over a 1-min period by measuring  $^{14}\text{C}$  uptake in 5-, 10-, 20-, 40-, and 60-s incubations. Measurements of intracellular C pools and photosynthetic kinetics were performed in 10-s experiments that used 10 external C concentrations ranging from  $\sim 10$  to 1000  $\mu\text{M}$ . Apparent photosynthetic half-saturation constants ( $K_m$ ) were estimated by least squares fitting of the Michaelis-Menten equation to the cellular organic  $^{14}\text{C}$  versus external DIC (Sigma plot; nonlinear regression algorithm). Below, we report only the results obtained with phytoplankton samples that showed significant physiological activity in short-term

experiments. Net  $\text{O}_2$  evolution and 10-s  $^{14}\text{C}$  uptake could not be detected in roughly a third of the samples we examined.

**Inhibitor studies**—To examine the role of carbonic anhydrase (CA) in photosynthetic C uptake and fixation, cells were treated with 100  $\mu\text{M}$  of the membrane permeable CA inhibitor Ethoxycarbonyl diethylammonium salt (EZ; Sültemeyer 1998) and short-term  $^{14}\text{C}$  experiments were performed as described above, with the exception that a single external C concentration was used (50  $\mu\text{M}$ ). Rates of photosynthetic  $\text{O}_2$  evolution were also measured in control and EZ-treated cells. These latter experiments, performed by use of the  $\text{O}_2$  electrode, were run in full C (i.e.,  $\sim 2.2 \text{ mM}$ ) seawater. The voltage output of the oxygen electrode was calibrated by use of solutions of air-equilibrated ( $\sim 230 \mu\text{M O}_2$ ) and  $\text{N}_2$ -purged ( $\sim \text{O}_2$ -free) seawater.

**Enzyme expression**—A number of phytoplankton samples filtered directly from Niskin bottles were frozen in liquid  $\text{N}_2$  for subsequent laboratory analysis of CA expression by Western blot analysis (Sambrooke et al. 1989). Frozen polycarbonate filters were thawed and plankton resuspended in  $\sim 1$  ml of a lysis buffer (50 mM NaCl, 50 mM  $\text{PO}_4^-$  pH 7.5 and 0.5% (v/v) Triton X-100). Samples were sonicated for five 30-s cycles (40% duty cycle) separated by 30-s pauses. Low biomass samples were concentrated by ultrafiltration with a 10,000 Dalton cutoff membrane. Cell extracts were diluted into a PAGE buffer (50 mM Tris base pH 6.8, 2% SDS, 10% glycerol, 5%  $\beta$ -mercaptoethanol) and boiled for 5 min. Total protein samples were electrophoresed through 12% SDS polyacrylamide gels, transferred to a polyvinylidene difluoride (PVDF) membrane, and blotted with a diatom-specific CA antiserum raised against *Thalassiosira weissflogii* protein (Roberts et al. 1997). The antiserum has been shown to cross-react widely with diatoms CAs (including proteins isolated from *Skeletonema*, *Phaeodactylum*, *Biddulphia*, *Nitzschia*, *Chaetoceros*, *Rhizosolenia*, *Cylindrotheca*, and *Coscinodiscus*) but not significantly with CAs from other phytoplankton taxa (chlorophytes, prymnesiophytes, rhodophytes, or cyanophytes; T. Lane, unpublished data). Cross reactivity with the CA immunoprobe was visualized by phosphorimaging blots exposed to  $^{125}\text{I}$ -labeled protein A (NEN-Dupont, 4066 Ci  $\text{mmol}^{-1}$ ).

For some samples, carbonic anhydrase activity was measured by use of a potentiometric assay that follows the pH change resulting from the hydration of  $\text{CO}_2$  in an alkaline buffer solution. Sonicated cell lysates (200  $\mu\text{l}$ ) were added to 3 ml of ice cold Veronal buffer solution (pH 8.4; 0.6 M Sorbitol) and the pH recorded every 5 s after the injection of 2 ml of  $\text{CO}_2$  saturated water ( $4^\circ\text{C}$ ). Blanks were run with 200  $\mu\text{l}$  milli-Q water and with samples preincubated for 2 min with 100  $\mu\text{M}$  EZ.

**Incubation experiments**—Incubation experiments were run to examine the steady-state physiological responses of phytoplankton assemblages to  $\text{CO}_2$  manipulations. Unfiltered seawater, collected from the upper mixed layer by use of a trace metal-clean in situ pumping system, was dispensed into acid-washed polycarbonate bottles. For one of the six incubations (Sta. P4 NE Pacific; 1998d in Table 1), nutrients were added (30  $\mu\text{M NO}_3^-$ , 10  $\mu\text{M SiO}_3^{2-}$ , 1  $\mu\text{M PO}_4^{3-}$ ) to promote phytoplankton growth. Most experiments were done with 2-liter bottles, but 4- and 20-liter containers were also

Table 1. Effects of CO<sub>2</sub> manipulations on the steady-state growth rates of coastal Pacific phytoplankton populations.

Date and location	Incubation time (d)	CO <sub>2</sub> level (ppm)*	Growth rate (d <sup>-1</sup> ) (±) SD
June 1996, Central California†	2.9	100	0.97 ± 0.05 (n = 2)
		350	0.96 ± 0.07
		800	0.94 ± 0.07
June 1997, Big Sur Coast	4.6	100	0.62 ± 0.10 (n = 2)
		350	0.45 ± 0.03
		800	0.62 ± 0.10 (n = 2)
May 1998a, Monterey Canyon	2	100	1.68 ± 0.21‡ (n = 2)
		350	2.45 ± 0.15
		800	2.30 ± 0.18
May 1998b, Central California	4.65	100	1.61 ± 0.05 (n = 2)
		350	1.63 ± 0.05
		800	1.67 ± 0.04
May 1998c, Central California	2.1	100	1.57 ± 0.04 (n = 3)
		800	1.67 ± 0.03
June 1998d, Sta. P4: NE Pacific Ocean	4.4	270	0.54 ± 0.06 (n = 3)
		750	0.53 ± 0.04

\* Water temperatures in incubation bottles averaged 13°C such that 100 ppm CO<sub>2</sub> ~ 3 μM, 350 ppm ~ 14 μM, 750 ppm ~ 30 μM, and 800 ppm ~ 32 μM.

† Data from Tortell et al. (1997).

‡ Statistically significant difference between 100 and 350 ppm treatment (*t* test; *P* < 0.05).

used. Samples were incubated on deck in a flow-through seawater chamber and covered with sufficient neutral density screening to reduce light intensity to ~30% of surface irradiance levels (i.e., ~600 μmol photons m<sup>-2</sup> s<sup>-1</sup>). Commercially prepared air mixtures containing 100, 270, 350, 750, or 800 ppm CO<sub>2</sub> were bubbled into incubation bottles to obtain aqueous CO<sub>2</sub> concentrations ranging between ~4 and 32 μM (0.2 μm in-line filters were used on gas lines to minimize particulate contamination of samples). As judged by periodic pH measurements, CO<sub>2</sub> concentrations in bottles equilibrated with the inflowing gas stream within 12–24 h and remained relatively constant over the course of the incubations (± < 10%). In the 100 and 800 ppm CO<sub>2</sub> treatments, seawater pH reached steady-state values of ~8.5 and 7.8, respectively, with corresponding CO<sub>3</sub><sup>2-</sup> ion concentrations of 390 and 95 μM (calculated from the equations of Millero 1996). Total DIC concentrations (i.e., CO<sub>2</sub> + HCO<sub>3</sub><sup>-</sup> + CO<sub>3</sub><sup>2-</sup>) were ~15% higher in the 800 ppm treatment relative to the 100 ppm treatment (2225 μM vs. 1902 μM), whereas alkalinity remained unchanged by the CO<sub>2</sub> manipulations. Note that our experimental perturbations of the DIC chemistry in seawater samples mimic those associated with photosynthetic CO<sub>2</sub> depletion as well anthropogenic CO<sub>2</sub> increases.

At least once per day, 100–200-ml samples were removed from incubation bottles (by use of positive pressure) for fluorometric measurements of total community (>0.2 μm) Chl *a* (Parsons et al. 1984). At the beginning and end of incubations, size-fractionated Chl *a* measurements were also made (>0.2, >5.0, and >18.0 μm). Net growth rates were calculated from regressions of the natural logarithm of phytoplankton biomass (Chl *a*) against time, with standard errors of regression coefficients derived from pooled replicate data. Incubations were run with duplicate or triplicate CO<sub>2</sub> treatments and terminated shortly before major nutrients were depleted from bottles.

For two of the 1998 Monterey Bay incubations, (1998b

and 1998c in Table 1), short-term <sup>14</sup>C uptake experiments (silicone oil centrifugation) were performed on concentrated cell suspensions from each CO<sub>2</sub> treatment (pooled replicates) on the final day of phytoplankton growth. In these experiments, only organic (acid-stable) C was measured, and <sup>14</sup>C in cell pellets was normalized to Chl *a* concentrations rather than biovolume. In addition, a number of samples were collected from these incubations for subsequent laboratory analysis. Expression levels of carbonic anhydrase and Rubisco were measured by Western blotting, as described above, with total phosphorimage counts (corrected for background blanks) normalized to total protein in cell extracts (determined with a modified Lowry assay; Pierce Chemical). The Rubisco antibody, kindly provided by Dr. Paul Falkowski, was raised against a chromophytic algae (*Isochrysis galbana*; see Falkowski et al. 1989). C:N ratios of phytoplankton collected on precombusted GF/C filters (4 h, 425°C) were measured by use of a Carlo-Erba elemental analyzer, and total carbohydrate and protein determined as described by Parsons et al. (1984).

*Stable C isotopes*—Particulate organic carbon (POC) samples from incubations 1998b and 1998c were collected on combusted GF/C filters on the final day of experiments and the <sup>13</sup>C/<sup>12</sup>C composition measured on an isotope ratio mass spectrometer and converted to conventional δ<sup>13</sup>C values, as previously described (Rau et al. 1990). HgCl<sub>2</sub>-poisoned samples for δ<sup>13</sup>C<sub>DIC</sub> analysis were taken from all CO<sub>2</sub> treatments at the beginning and end of the incubations. DIC samples were acidified under vacuum, and the liberated CO<sub>2</sub> was trapped cryogenically and analyzed by mass spectrometry. The isotopic composition of aqueous CO<sub>2</sub> in samples (δ<sup>13</sup>C<sub>CO<sub>2</sub>aq</sub>) was calculated from the measurements of δ<sup>13</sup>C<sub>DIC</sub> that used the equations of Mook et al. (1974). Because the isotopic composition of CO<sub>2</sub> in mixed gas tanks (~-40‰) differed significantly from that in the initial seawater samples (~-9‰), δ<sup>13</sup>C<sub>CO<sub>2</sub>aq</sub> in incubation bottles changed significantly over the course of the bubbling experiments. To

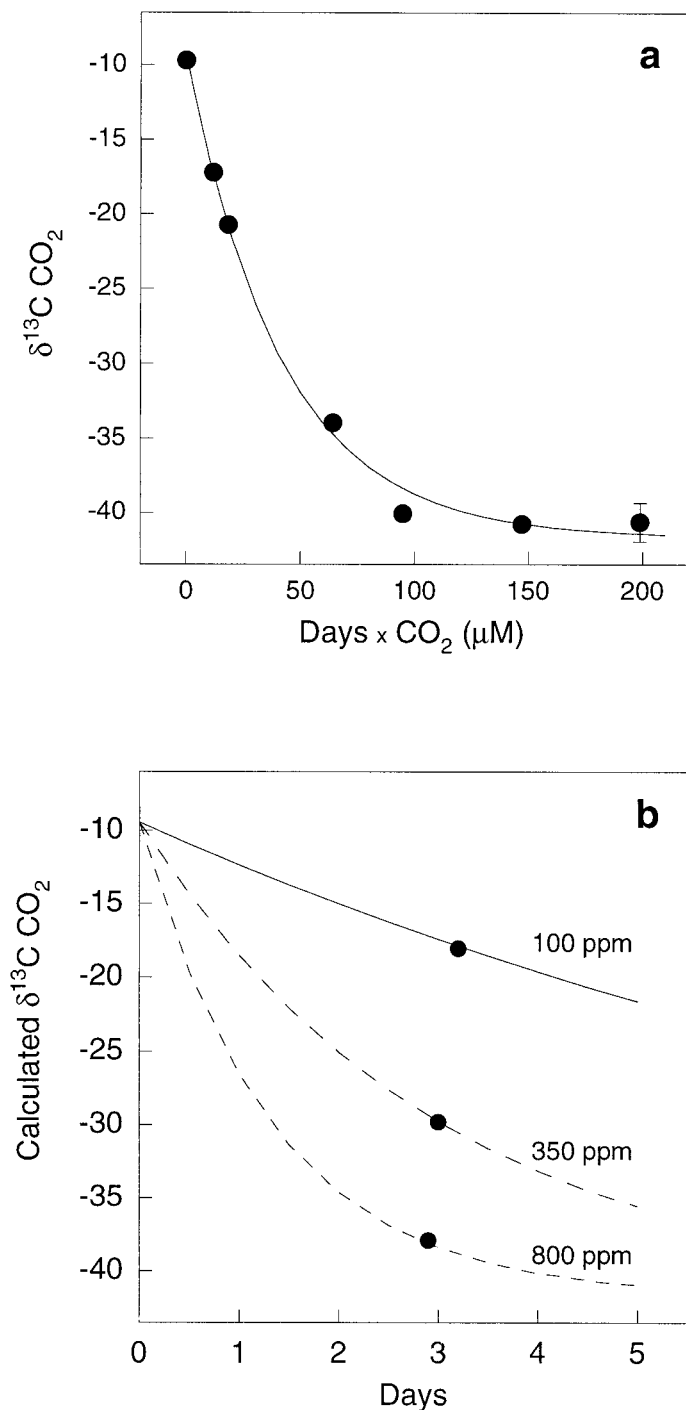


Fig. 1. Data and modeling used to estimate temporal changes in  $\delta^{13}\text{C}_{\text{CO}_2\text{aq}}$  during  $\text{CO}_2$ -controlled incubation experiments. (a) Variation in final  $\delta^{13}\text{C}_{\text{CO}_2\text{aq}}$  values (calculated from measured  $\delta^{13}\text{C}_{\text{DIC}}$ ) across incubations as a function of inflowing  $\text{CO}_2$  concentration and total bubbling time. To estimate the rate of isotopic equilibration in samples, an exponential decay curve was fitted to the data ( $\delta^{13}\text{C}_{\text{CO}_2\text{aq}} = 32.17 \times \exp(-0.024 \times \text{time}) - 31.33$ ; root mean square error = 0.86‰,  $r^2 = 0.98$ ,  $P < 0.01$ ). Error bars represent standard errors of the means and are smaller than symbol where not seen. (b) The equilibration rate constant  $k$  ( $= -0.024$ ) is used to predict the time-dependent changes in  $\delta^{13}\text{C}_{\text{CO}_2\text{aq}}$  for each  $\text{CO}_2$  treatment. To estimate the effective  $\delta^{13}\text{C}_{\text{CO}_2\text{aq}}$  experienced by phytoplank-

ton in the incubations ( $\delta^{13}\text{C}_{\text{CO}_2\text{eff}}$ , filled symbol on curves), predicted  $\delta^{13}\text{C}_{\text{CO}_2\text{aq}}$  values are weighted by phytoplankton biomass (calculated from measured growth rates), averaged over 1-hr intervals, and summed over the total incubation time.

## Results

*In situ samples*—Short-term  $^{14}\text{C}$  uptake and fixation were measured to examine the cellular DIC pool sizes and photosynthetic kinetics of natural phytoplankton assemblages. Time-course experiments run with four different assemblages (at 100  $\mu\text{M}$  external DIC) showed that internal inorganic C pools rapidly reached steady-state values (i.e.,  $< 5$  s), whereas cellular organic C accumulated linearly over time (Fig. 2). Acid-stable (organic) C accounted for between 1% and 15% of total cellular C pools after 5 s and 15%–30% after 60 s.

In 10-s  $^{14}\text{C}$  uptake experiments over a range of external DIC from  $\sim 10$  to 1000  $\mu\text{M}$ , phytoplankton from three independent coastal populations showed a similar linear increase in intracellular DIC, with only a faint indication of saturation at the higher external C concentrations tested (Fig. 3a). On the basis of the time-course experiments (Fig. 2), we assume that cellular inorganic C measured after 10 s represents a steady-state pool. In all three assemblages, intracellular concentrations of DIC were significantly higher ( $t$ -test;  $P < 0.01$ ) than those in the experimental medium (see 1:1 line on figure). Inorganic carbon concentration factors (CCFs)—defined as the ratio of internal DIC to extracellular DIC (calculated as the slopes of linear regressions fitted to the data in Fig. 3a) ranged from  $\sim 3$  to 5.5 (average  $\sim 4.5$ ).

The accumulation of cellular inorganic C was associated with significant photosynthetic C fixation in all experiments. Phytoplankton from the three assemblages showed a similar relationship between C fixation rates and external  $\text{DI}^{14}\text{C}$  levels in the test solution (Fig. 3b). The best-fit Michaelis-Menten curve (nonlinear regression by use of data from all experiments) yielded an apparent photosynthetic half-saturation constant ( $K_m$ ) of 240 ( $\pm 85$ ; SE)  $\mu\text{M}$  external DIC or  $\sim 1.2$  ( $\pm 0.42$ )  $\mu\text{M}$  external  $\text{CO}_2$  ( $\text{CO}_2 \sim 0.5\%$  of DIC at pH 8.2; Millero 1996).

The membrane-permeable carbonic anhydrase inhibitor EZ had a substantial effect on C fixation by the natural phytoplankton assemblages. (Note that the phytoplankton used

←

ton in the incubations ( $\delta^{13}\text{C}_{\text{CO}_2\text{eff}}$ , filled symbol on curves), predicted  $\delta^{13}\text{C}_{\text{CO}_2\text{aq}}$  values are weighted by phytoplankton biomass (calculated from measured growth rates), averaged over 1-hr intervals, and summed over the total incubation time.

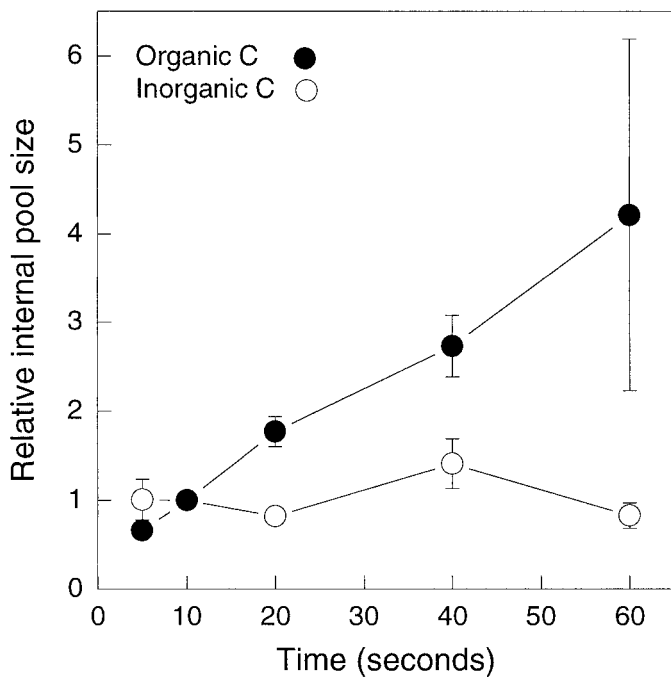


Fig. 2. Time course of  $^{14}\text{C}$  uptake into cellular organic and inorganic pools in four Pacific phytoplankton assemblages. Cells were collected in situ and C uptake measured by use of the silicone oil centrifugation technique ( $100\ \mu\text{M}$  external C). All  $^{14}\text{C}$  counts for each assemblage are normalized to the values obtained in 10-s incubations that are as follows: Experiment 1: 389 dpm organic  $^{14}\text{C}$ , 18,988 dpm inorganic  $^{14}\text{C}$ ; Experiment 2: 1214 dpm organic  $^{14}\text{C}$ , 5511 dpm inorganic  $^{14}\text{C}$ ; Experiment 3: 859 dpm organic  $^{14}\text{C}$ , 4112 dpm inorganic  $^{14}\text{C}$ ; and Experiment 4: 252 dpm organic  $^{14}\text{C}$ , 2272 dpm inorganic  $^{14}\text{C}$ . Error bars represent standard errors of the pooled data from all experiments and are smaller than symbols where not seen.

in EZ inhibition experiments [Fig. 4] were not the same as those used in the C uptake studies shown in Fig. 3.) In two independent experiments, the addition of  $100\ \mu\text{M}$  EZ to concentrated cell suspensions resulted in a significant decrease in photosynthetic rates as measured by steady-state (2–3 min)  $\text{O}_2$  evolution in full C seawater or short-term  $^{14}\text{C}$  fixation in low C buffer (Fig. 4a,b). Despite the strong negative effect of EZ on photosynthesis, the inhibitor did not significantly affect the steady-state concentration of cellular inorganic C (Fig. 4b).

Evidence of CA expression in situ by phytoplankton populations was obtained from Western blots of samples collected from Niskin bottles. Cross-reactivity with the diatom-specific CA antibody was found in two of seven samples collected from the 1997 Monterey Bay cruise (Fig. 5a). The putative CA appeared as a protein band on polyacrylamide gels whose apparent molecular weight was very similar to that of the *T. weissflogii* positive control ( $\sim 28\ \text{kDa}$ ). (Note that negative Western Blot results do not rule out CA expression by the phytoplankton. CA sequences exhibit significant phylogenetic diversity, and the immunoprobe raised against *T. weissflogii* may react poorly with the proteins of some indigenous phytoplankton species.) In two of five samples collected from the North Pacific California Current, sig-

nificant CA activity was detected in potentiometric assays of cell lysates (Fig 5b). (As with the Western blot analysis, negative results in the potentiometric assay do not necessarily rule out CA expression by the phytoplankton in situ.)

**Incubation studies**—Figure 6 shows a representative set of growth curves taken from a Monterey Bay  $\text{CO}_2$  manipulation experiment (1998b), whereas Table 1 presents a summary of the growth-rate data for six incubations (1996–1998). Growth rates of the phytoplankton assemblages ranged from  $0.45$  and  $2.45\ \text{d}^{-1}$  and were highly reproducible among replicate treatment bottles (average SE  $< 10\%$  of the means). In five of the six experiments, growth rates were constant across  $\text{CO}_2$  treatments. The one exception was an incubation conducted in Monterey Bay waters (incubation 1998a), in which growth was significantly inhibited (30% reduction) at the lowest  $\text{CO}_2$  level tested ( $100\ \text{ppm}$ ) but showed no statistical difference between the intermediate ( $350\ \text{ppm}$ ) and high ( $800\ \text{ppm}$ )  $\text{CO}_2$  treatments.

The physiological and biochemical responses of Monterey Bay phytoplankton to  $\text{CO}_2$  manipulations were examined for two 1998 experiments (1998b and 1998c). The algal assemblages in these incubations were dominated by large diatoms ( $> 60\%$  retained on  $18\ \mu\text{m}$  filters), with prevalent species including representatives of *Asterionella*, *Chaetoceros*, *Thalassiosira*, and *Skeletonema* (incubation 1998b) and *Nitzschia* (incubation 1998c). For both incubations, no apparent  $\text{CO}_2$ -dependent differences in species composition could be detected by either microscopic examination or size-fractionated Chl *a* measurements of samples collected at the end of the growth period. We therefore assume that population level changes do not contribute significantly to the observed physiological responses of phytoplankton assemblages to  $\text{CO}_2$  manipulations reported below.

Although the  $\text{CO}_2$  manipulations had no discernible effect on the steady-state growth rates of phytoplankton in the Monterey Bay 1998b and 1998c incubations (Table 1), short-term (10 s)  $^{14}\text{C}$  fixation rates, measured on the final day of experiments, differed systematically among  $\text{CO}_2$  treatments (Fig. 7). Cells preconditioned at low  $\text{CO}_2$  ( $100\ \text{ppm}$ ) assimilated organic  $^{14}\text{C}$  at significantly faster rates than those grown with high  $\text{CO}_2$  ( $800\ \text{ppm}$ ) when exposed to the same (nonsaturating) external  $\text{DI}^{14}\text{C}$  (*t*-test;  $P < 0.05$ ). In incubation 1998b, the rate of  $^{14}\text{C}$  uptake in  $350\ \text{ppm}$  preconditioned phytoplankton was intermediate between that of the  $100\ \text{ppm}$ - and  $800\ \text{ppm}$ -acclimated cells (see Fig. 7 legend for regression slopes  $\pm$  standard errors of photosynthetic rates vs. external DIC).

The effects of  $\text{CO}_2$  preconditioning on short-term  $^{14}\text{C}$  fixation rates were mirrored by changes in the cellular levels of carbonic anhydrase and Rubisco (as measured by Western blot analysis), both of which showed enhanced expression in low  $\text{CO}_2$  grown phytoplankton (Fig. 8). (Given that phytoplankton species composition did not appear to differ significantly across  $\text{CO}_2$  treatments, it is unlikely that differential enzyme expression resulted from variable cross-reactivity of the immunoprobe.) Bulk cellular biochemical composition was also influenced to some extent by  $\text{CO}_2$  levels. In the 1998b incubation, ratios of C to N and carbohydrate to protein decreased significantly in the lowest  $\text{CO}_2$  treatment but did not differ significantly between  $350$  and  $800\ \text{ppm}$ -grown cells (Fig. 9). Increasing C:N at high  $\text{CO}_2$

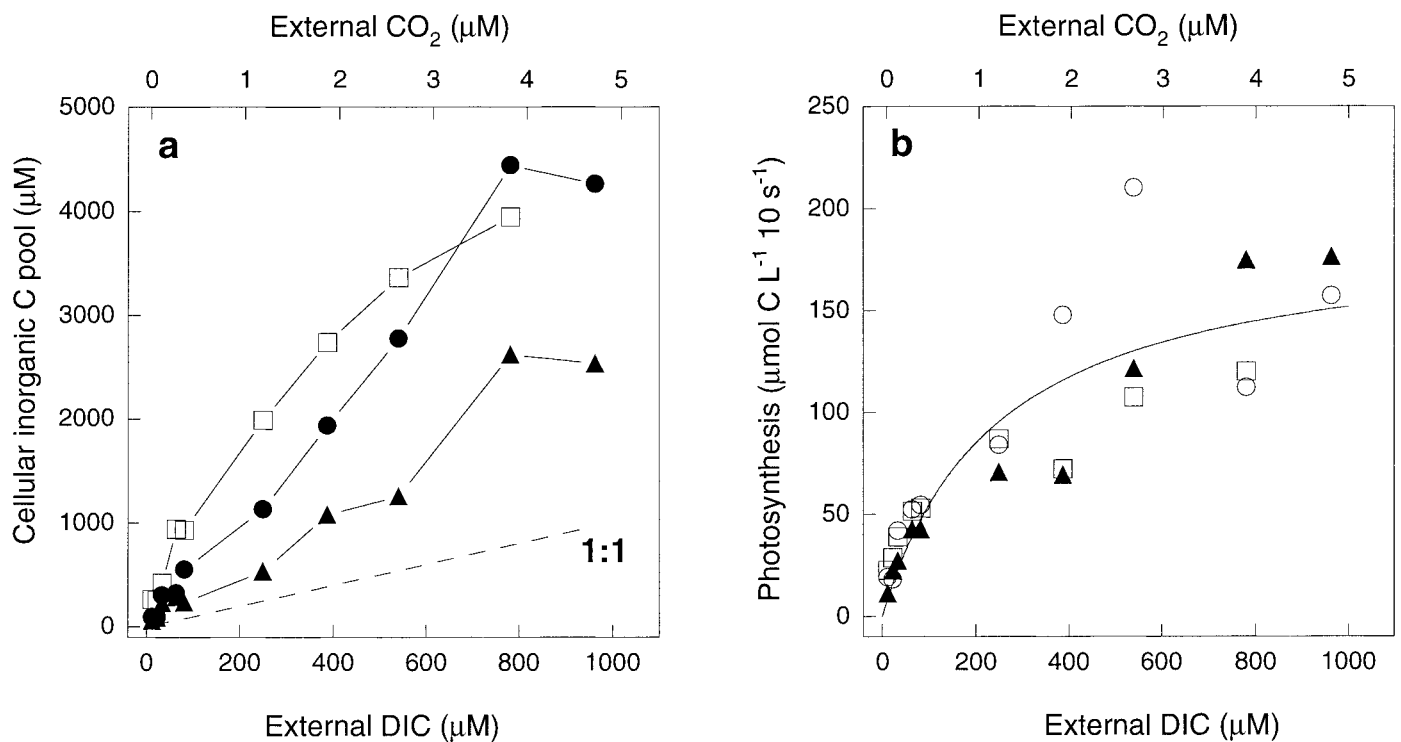


Fig. 3. Short-term photosynthetic kinetics and intracellular C concentrations in three Pacific Ocean phytoplankton assemblages measured by use of the silicone oil centrifugation technique (pH 8.2). (a) Cellular concentrations of inorganic (acid labile) C accumulated in 10-s experiments at external DIC concentrations ranging from  $\sim 10$  to  $1000 \mu\text{M}$ . For all three assemblages (each denoted by a different symbol), intracellular inorganic C concentrations were significantly higher (3–5.5-fold) than those in the test solution (see 1:1 dashed line on figure). (b) Photosynthetic rates of the assemblages as measured by organic (acid-stable) C accumulated in 10 s. The apparent half-saturation constant derived from a nonlinear regression of the Michaelis-Menten equation is  $240$  (SE =  $\pm 85$ )  $\mu\text{M}$  total DIC or  $1.2$  ( $\pm 0.42$ )  $\mu\text{M}$   $\text{CO}_2$  ( $r^2 = 0.79$ ,  $P < 0.01$ ).

was associated with an increase in cellular carbohydrate relative to protein (Fig. 9). For incubation 1998c, no  $\text{CO}_2$ -dependent effects were evident on bulk biochemical composition. Note however, that this experiment was significantly shorter than the 1998b incubation ( $\sim 2$  d, compared with  $\sim 4.5$  d). No effect of  $\text{CO}_2$  on C:Chl *a* ratios was seen in either incubation experiment ( $45.0 \pm 2.1$  mol C: mol Chl *a*).

**C isotope fractionation**—The C isotopic composition of phytoplankton ( $\delta^{13}\text{C}_{\text{POC}}$ ) collected at the end of incubation experiments ranged from  $-20.1\text{‰}$  to  $-45.3\text{‰}$  (Table 2). Biological fractionation factors ( $\epsilon_p$ ), calculated by use of the  $\delta^{13}\text{C}_{\text{CO}_2\text{eff}}$  values (see Materials and Methods), varied between  $3.44\text{‰}$  and  $7.21\text{‰}$  (average SE =  $6.1\text{‰} \pm 0.45\text{‰}$ ) and were not significantly correlated to  $\text{CO}_2$  levels across treatments ( $P > 0.1$ ). Phytoplankton in the 1998b incubation exhibited slightly higher C-isotope fractionation ( $\epsilon_p$ :  $4.97\text{‰}$ – $7.21\text{‰}$ ) than those from incubation 1998c ( $\epsilon_p$ :  $3.44\text{‰}$ – $4.60\text{‰}$ ). The variability in  $\epsilon_p$  between experiments could not be accounted for by differences in growth rates, which were negligible (see Table 1) and may reflect differences in the species composition of the phytoplankton assemblages in the incubations.

## Discussion

This field study was conducted to investigate inorganic C acquisition in coastal marine phytoplankton assemblages and

the physiological responses of cells to environmentally relevant  $\text{CO}_2$  manipulations. Our results indicate that the diatom-dominated plankton communities we studied used carbon concentrating mechanisms that are modulated by ambient  $\text{CO}_2$  concentrations. The phytoplankton assemblages possessed the capacity to concentrate internal inorganic C pools, showed a high affinity for external inorganic C, expressed carbonic anhydrase, and fractionated stable C isotopes in a manner inconsistent with purely diffusive  $\text{CO}_2$  uptake. Although steady-state growth rates were generally unaffected by  $\text{CO}_2$  manipulations, the  $\text{CO}_2$ -dependent regulation of C acquisition affected the biochemical composition of cells. Our data show that regulated C uptake mechanisms are important for the growth of (at least some) phytoplankton in coastal marine waters.

**Inorganic C acquisition**—The results we obtained in our study of C acquisition in coastal Pacific phytoplankton are consistent with previous laboratory and field work on diatom cultures and diatom-dominated natural assemblages. The apparent cellular carbon concentrating factor we measured in short-term  $^{14}\text{C}$  uptake experiments ranged from 3 to 5.5 (average 4.5) for three different Pacific assemblages (Fig. 3a). Using similar experimental techniques, we previously measured a  $\sim 10$ -fold C concentration factor in Delaware Bay diatom populations (Tortell et al. 1997). Earlier culture studies of several diatom species have reported intracellular C

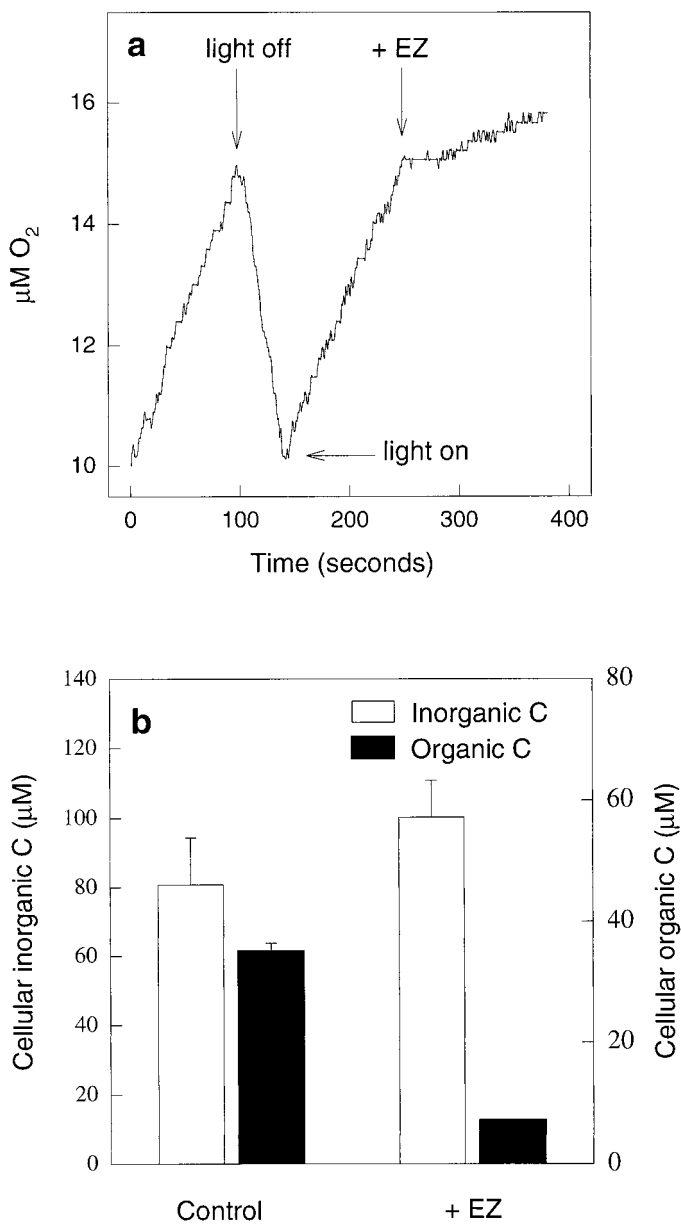


Fig. 4. The effects of carbonic anhydrase inhibitors on photosynthesis and short-term inorganic C uptake and assimilation in two distinct phytoplankton assemblages. (a) Oxygen evolution rates of a coastal assemblage in the presence and absence of 100  $\mu\text{M}$  EZ—a membrane permeable carbonic anhydrase inhibitor. Rates were measured in an  $\text{O}_2$  electrode in full C seawater ( $\sim 2.2$  mM). Arrows on the figure designate the times when the light source (slide projector) was turned off and on and when the inhibitor was added to the cell suspension. (b) Short-term  $^{14}\text{C}$  accumulation into inorganic (acid-labile) and organic (acid-stable) cellular pools in the presence and absence of 100  $\mu\text{M}$  EZ. Measurements were made in 10-s silicone oil centrifugation experiments. Error bars represent standard errors of the means. Only the organic C pool differs significantly ( $t$ -test;  $P < 0.05$ ) between treatments.

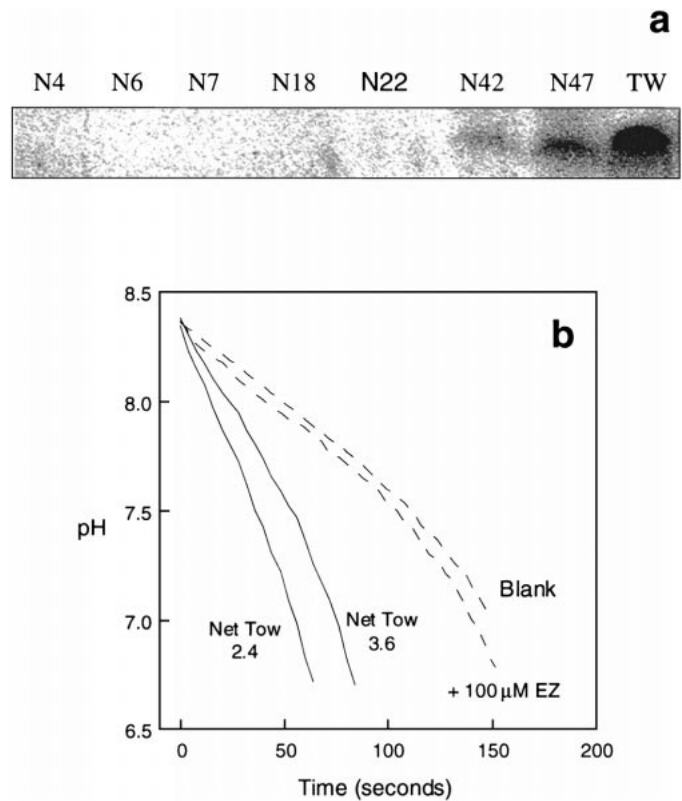


Fig. 5. In situ carbonic anhydrase expression by coastal Pacific phytoplankton assemblages. (a) Western blot cross reactivity of coastal phytoplankton samples with a carbonic anhydrase antibody raised against the centric diatom *T. weissflogii*. Codes above sample lanes correspond to Niskin bottle casts, whereas the far right lane contains a *T. weissflogii* positive control. (b) Potentiometric assay of carbonic anhydrase expression in plankton net tow samples from the California current. The rate of pH change is proportional to the rate of  $\text{CO}_2$  hydration, which is enhanced by the presence of carbonic anhydrase relative to blank samples or those treated with CA inhibitors. Note that different phytoplankton samples were used for Western blots and potentiometric assays.

pools ranging from 2- to 25-fold higher than external DIC levels (see Badger et al. 1998). Note that our field measurements of cellular C accumulation factors were made at external DIC concentrations  $\leq 1$  mM and may thus overestimate C accumulation ratios at seawater levels of DIC ( $\sim 2.2$  mM). However, because we did not observe saturation of cellular inorganic C pools at 1 mM, it seems likely that cells growing in full C seawater should have internal DIC concentrations that are equal to or greater than those we measured in our experiments and thus still show significant cellular C accumulation relative to the external environment.

Several caveats should be considered when interpreting data on cellular C accumulation in phytoplankton. Estimates of C concentrating factors assume that C is distributed homogeneously within the cell (i.e., the CCF is calculated by normalizing measured C pools to total cell volume). This calculation may underestimate  $\text{CO}_2$  concentrations at the site of carboxylation by Rubisco if C is accumulated preferentially into chloroplasts or subchloroplastic compartments such as pyrenoids. Studies with green algae suggest that

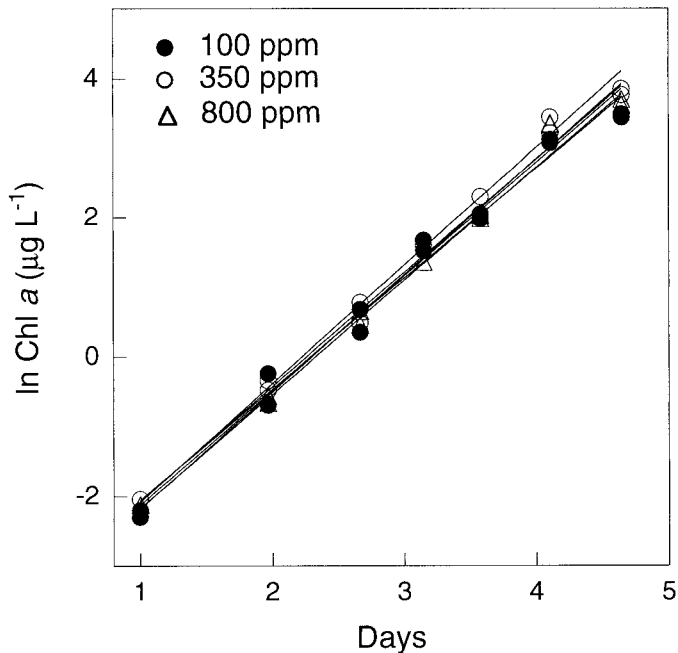


Fig. 6. Effects of  $\text{CO}_2$  manipulations on steady-state growth rates of Monterey Bay phytoplankton. Total community ( $>0.2 \mu\text{m}$ ) Chl *a* was measured. Lines are least square linear regressions.

chloroplasts play a role in active C uptake and that subcellular compartmentalization of inorganic C may indeed occur in at least some phytoplankton (Moroney and Chen 1998). Furthermore, the chemical speciation of inorganic C in the cell—i.e., the fraction existing as free  $\text{CO}_2$ —is likely to differ from that in seawater, given the difference in pH that exists between the cellular and external environments. This assertion assumes chemical equilibrium among cellular DIC species, which may or may not exist depending on the rate at which a particular species is added to and removed from a given DIC pool and the presence and localization of carbonic anhydrase (see below). Despite these complexities, the cellular accumulation of inorganic C by the coastal phytoplankton assemblages provides prima facie evidence that they possess some form of carbon concentrating mechanism.

The photosynthetic kinetics of the Pacific phytoplankton assemblages are also indicative of a CCM. The apparent cellular half-saturation constant ( $K_m$ ) in short-term photosynthesis experiments was  $\sim 240$  (SE =  $\pm 85$ )  $\mu\text{M}$  DIC or  $\sim 1.2$  ( $\pm 0.42$ )  $\mu\text{M}$   $\text{CO}_2$  (Fig. 3b). This value agrees well with our previous results from Delaware Bay ( $\sim 2.5 \mu\text{M}$   $\text{CO}_2$ ; Tortell et al. 1997) and with the  $K_{m(\text{CO}_2)}$  measurements reported for a number of phytoplankton known to possess C concentrating mechanisms ( $0.65 \pm 0.5$ ; see review by Raven and Johnston 1991). For most species thus far examined, apparent cellular  $\text{CO}_2$  affinities in vivo are significantly higher than

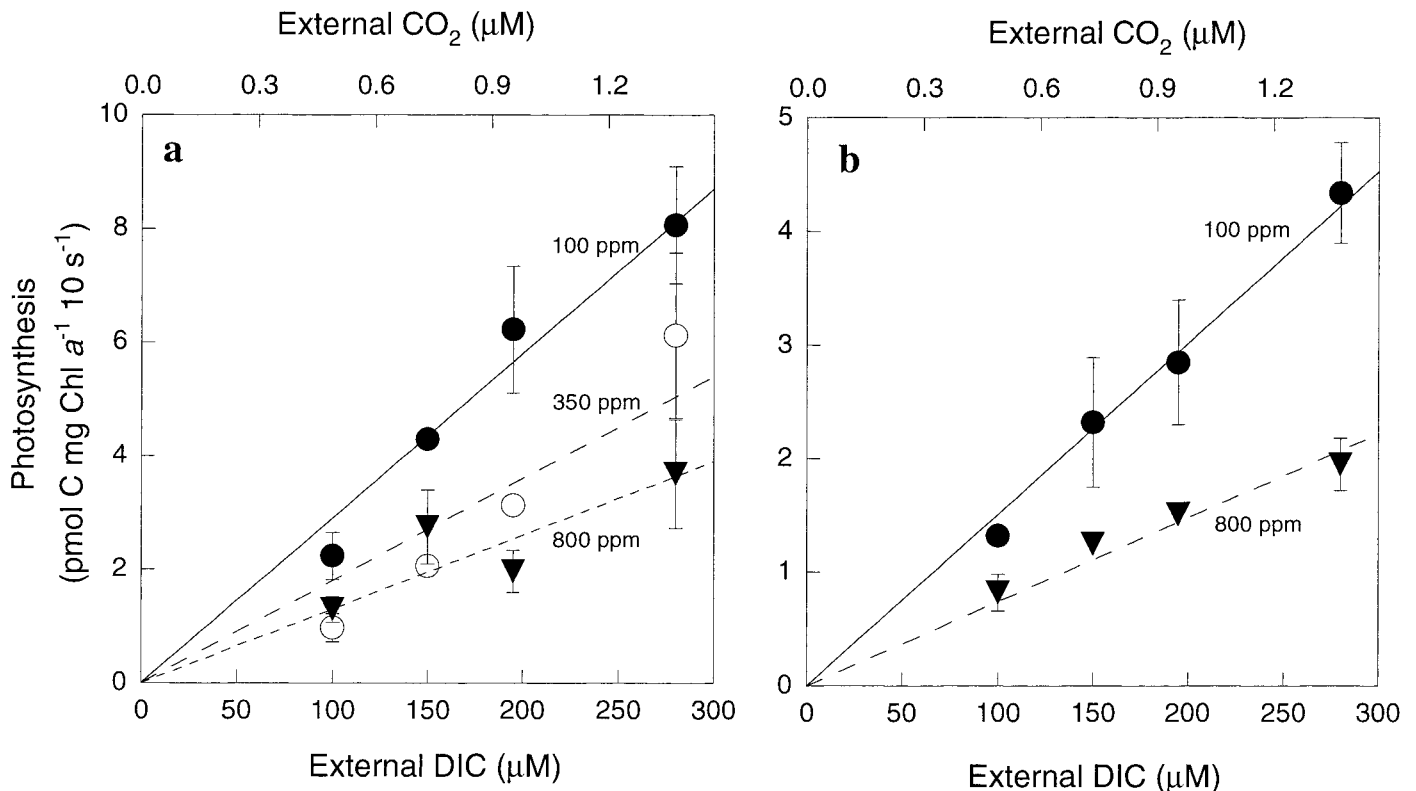


Fig. 7. Effects of  $\text{CO}_2$  manipulations on short-term  $^{14}\text{C}$  fixation by Monterey Bay phytoplankton. Rates of  $^{14}\text{C}$  assimilation into acid-stable cellular pools were measured in 10-s silicone oil centrifugation experiments on the final day of  $\text{CO}_2$ -controlled incubation experiments. (a) Incubation 1998b (see Table 1). Slopes ( $\pm$ SE) of the regression lines are 100 ppm,  $0.029 \pm 0.003$ ; 350 ppm,  $0.018 \pm 0.003$ ; and 800 ppm,  $0.013 \pm 0.002$  ( $\text{pmol C} \cdot \mu\text{g Chl a}^{-1} \cdot 10 \text{ s}^{-1} \cdot \mu\text{M external DIC}^{-1}$ ). (b) Incubation 1998c: 100 ppm,  $0.015 \pm 0.001$  and 800 ppm,  $0.0074 \pm 0.0001$ . All slopes are differ significantly (*t*-test;  $P < 0.05$ ) except for incubation 1998b 350 ppm and 800 ppm ( $P < 0.1$ ).

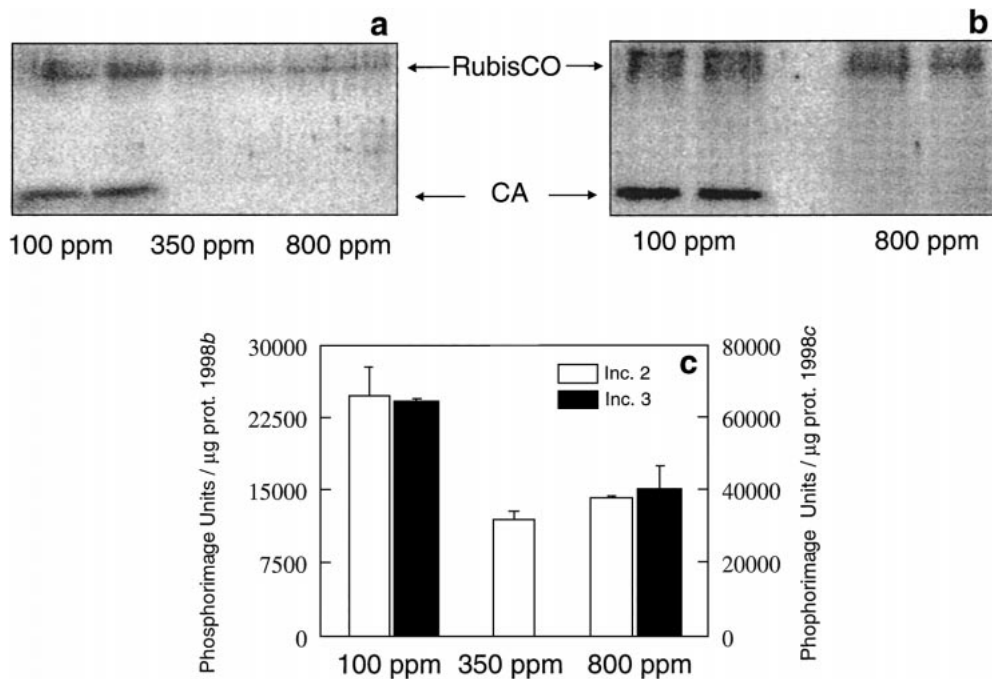


Fig. 8. Effects of CO<sub>2</sub> manipulations on expression levels of carbonic anhydrase and Rubisco in Monterey Bay phytoplankton. (a,b) Western blot of phytoplankton samples from incubations 1998b and 1998c, respectively, showing cross-reactivity with Rubisco and carbonic anhydrase immunoprobes. Samples were collected on the final day of incubations. Cross-reactivity is visualized by use of <sup>125</sup>I-labeled protein A, which reacts with bound antibodies. (c) Quantification of Rubisco and carbonic anhydrase expression normalized to total protein in loaded PAGE samples shown in (a) and (b). Error bars represent standard errors of means.

those of isolated Rubisco in vitro ( $K_m$  range for all phytoplankton, 20–200  $\mu\text{M}$ ; Badger et al. 1998). The discrepancy between in vivo and in vitro photosynthetic kinetics has been taken as evidence for the existence of a high intracellular carbon pool (Raven and Johnston 1991). The in vivo CO<sub>2</sub> affinity of the coastal (diatom-dominated) phytoplankton assemblages ( $K_m = 1.2 \mu\text{M}$ ) was ~25–50-fold higher than that of isolated diatom Rubisco ( $K_m$  range ~30–60  $\mu\text{M}$ ; Badger et al. 1998). The high apparent CO<sub>2</sub> affinity of the coastal phytoplankton relative to Rubisco can be explained by considering the elevated concentrations of inorganic C inside the cells. At an external DIC concentration of 240  $\mu\text{M}$  that half saturated photosynthesis by the assemblages (i.e., the apparent  $K_m$ ), total cellular inorganic C ranged between 500 and 2,000  $\mu\text{M}$  (Fig. 3a). Assuming an intracellular pH of 7.6 (Colman and Rotatore 1988; Mitchell and Beardall 1996) and chemical equilibrium among DIC species, this is equivalent to 12–50  $\mu\text{M}$  CO<sub>2</sub> in the cell. This range of intracellular CO<sub>2</sub> concentrations encompasses the half-saturation constant of diatom Rubisco (~30–60  $\mu\text{M}$ ) and would, therefore, be expected to yield approximately half-maximum rates of C fixation by the enzyme. At the highest external DIC concentrations we tested (1 mM), total intracellular DIC ranged between ~2,500 and 4,500  $\mu\text{M}$ , which, on the basis of the calculations outlined above, is equivalent to cellular CO<sub>2</sub> levels of ~60–110  $\mu\text{M}$ . Unless cellular C concentrations decrease significantly from 1 to 2 mM external C (which seems very unlikely), this analysis suggests that cells concentrate

cellular CO<sub>2</sub> significantly with respect to external CO<sub>2</sub> in seawater (~12  $\mu\text{M}$ ).

Although our field data on cellular C concentration factors and photosynthetic kinetics provide strong evidence for the operation of a CCM in coastal diatom populations, the specific molecular mechanisms underlying inorganic C transport and assimilation remain open to debate. Laboratory studies with a number of model phytoplankton species indicate that both HCO<sub>3</sub><sup>-</sup> and CO<sub>2</sub> can be actively taken up by cells (Raven 1997), although the transport systems have yet to be isolated and characterized at the molecular level. In some species, including a number of diatoms, active CO<sub>2</sub> uptake is believed to be coupled to an external (periplasmic) CA that maintains instantaneous equilibrium between HCO<sub>3</sub><sup>-</sup> and CO<sub>2</sub> in the vicinity of the cell (Sültemeyer 1998).

Although our measurements of CA expression in the phytoplankton assemblages (Fig. 5) provide no information on the cellular localization of the enzyme, our inhibitor studies (Fig. 4) suggest that it is essential for C fixation but not necessarily for C uptake. As we have previously observed in Delaware Bay phytoplankton (Tortell et al. 1997), the addition of EZ to coastal Pacific phytoplankton samples did not appear to affect cellular inorganic C accumulation (i.e., steady-state DIC pool size; Fig. 4). This result implies that some cells do not require periplasmic CA for C uptake and might directly transport HCO<sub>3</sub><sup>-</sup>, as reported for several species in laboratory cultures (Colman and Rotatore 1988, 1995). However, the modulation of the C transport system

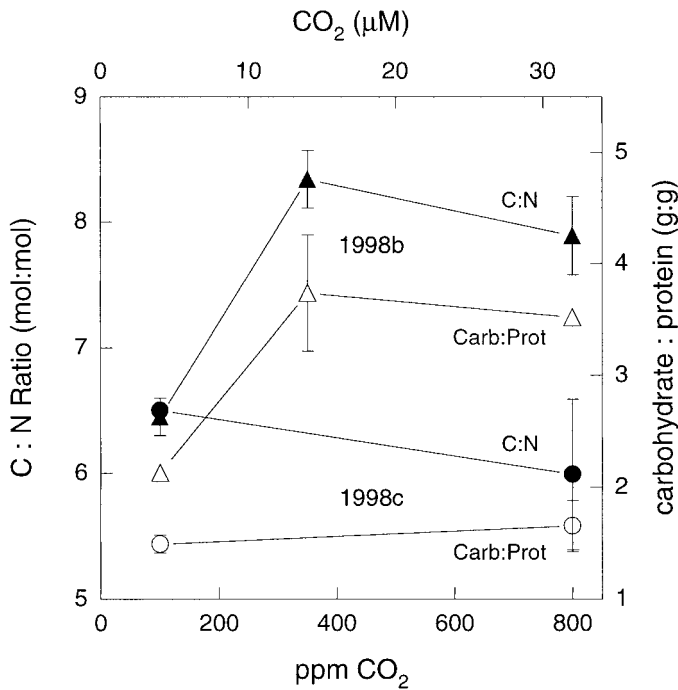


Fig. 9. Effects of CO<sub>2</sub> manipulations on biochemical compositions of Monterey Bay phytoplankton. Phytoplankton samples were collected on the final day of incubations 1998b and 1998c and C:N and carbohydrate:protein determined as outlined in Materials and Methods. Only the low and intermediate CO<sub>2</sub> treatments (100 ppm and 350 ppm) of incubation 1998b differ significantly (*t*-test; *P* < 0.05).

by CO<sub>2</sub> (Fig. 7) suggests that this inorganic C species is somehow involved in the active C uptake mechanism. According to most current models of C acquisition in phytoplankton, at least one form of intracellular CA is used to catalyze the conversion of HCO<sub>3</sub><sup>-</sup> to CO<sub>2</sub> for fixation by Rubisco in the chloroplast or by sub-chloroplastic compartments (Sültemeyer 1998). In contrast, very recent work has identified a novel pathway for C assimilation in diatoms through which CO<sub>2</sub> is converted to HCO<sub>3</sub><sup>-</sup> by a cytoplasmic CA and fixed via PEP-carboxylase into a C<sub>4</sub> compound (malic acid) that is decarboxylated in the chloroplast to yield CO<sub>2</sub> for Rubisco (Reinfelder et al. in press). Our results are not inconsistent with such a mechanism. The advent of more advanced molecular probes—both for CA and for putative HCO<sub>3</sub><sup>-</sup> and CO<sub>2</sub> transporters—is necessary before detailed

examinations of inorganic C assimilation in taxonomically diverse natural phytoplankton communities will be possible.

**CO<sub>2</sub> regulation of C acquisition and cellular biochemistry**—During the course of our 2–4 d incubations, Pacific diatom assemblages responded to variations in external CO<sub>2</sub> concentrations by modulating the affinity of an inorganic C transport system (Fig. 7), the expression of CA and Rubisco (Fig. 8), and, in one case, the cellular ratios of C:N and carbohydrate:protein (Fig. 9). These biochemical changes occurred, for the most part, in the absence of significant changes in steady-state growth rates. The CO<sub>2</sub>-dependent regulation of the C uptake system provides new and compelling evidence against purely diffusive CO<sub>2</sub> uptake by coastal phytoplankton assemblages.

Numerous laboratory studies have examined the regulation of the CCM by aqueous CO<sub>2</sub> in green algae, cyanobacteria, and, to a much lesser extent, in diatoms and coccolithophores (*see* Raven 1997 for a review). Cells that are transferred from high CO<sub>2</sub> conditions (10,000 ppm–30,000 ppm) to air levels of CO<sub>2</sub> (~350 ppm) show a dramatic increase in CA expression coupled to an increase in C affinity (i.e., decreased half-saturation constant) within 8–24 h. Recent work by Lane and Morel (2000a) has examined the modulation of diatom CA activity over a much narrower, environmentally relevant range of CO<sub>2</sub> levels (100 ppm–750 ppm).

In a field study of C acquisition by Lake Kinneret phytoplankton, Berman-Frank et al. (1998) documented the induction of a CCM in the dominant dinoflagellate *Peridinium gatunense* in response to declining CO<sub>2</sub> (increasing pH) over the course of a spring bloom. In this freshwater system, extensive primary production (>2000 mg C m<sup>-2</sup> d<sup>-1</sup>) causes a ~2-fold decrease in total inorganic C and a 20–50-fold reduction in aqueous CO<sub>2</sub> concentrations. By comparison, variations in the DIC chemistry of seawater are relatively small. In our experimental system, total DIC was nearly constant across treatments (~15% changes), whereas CO<sub>2</sub> levels varied by a factor of 8. Our field results therefore provide evidence that marine phytoplankton in situ may regulate inorganic C acquisition in response to variations in aqueous CO<sub>2</sub> rather than total DIC.

The co-regulation of CA and Rubisco in response to CO<sub>2</sub> concentrations (Fig. 8) has not been reported before in field studies, yet this result is not unexpected on the basis of the biochemistry of photosynthesis and C acquisition in phytoplankton. As an integral part of the CCM, CA acts to maintain a rapid rate of CO<sub>2</sub> supply to Rubisco (directly or in-

Table 2. Stable C isotope fractionation by incubated (Inc.) Monterey Bay phytoplankton.

Experimental treatment	δ <sup>13</sup> C POC (‰)	δ <sup>13</sup> C CO <sub>2</sub> effective (‰)	Fractionation ε <sub>p</sub> (‰)	μ <sub>i</sub> /CO <sub>2</sub> Kg μmol <sup>-1</sup> d <sup>-1</sup>
Inc. 2 100 ppm	-23.15 ± 0.08	-18.07 ± 0.24	4.97 ± 0.32	0.715
Inc. 2 350 ppm	-37.30 ± 0.57	-29.82 ± 0.45	7.21 ± 1.02	0.206
Inc. 2 800 ppm	-45.25 ± 0.08	-37.94 ± 0.44	6.99 ± 0.52	0.093
Inc. 3 100 ppm	-20.05 ± 0.07	-15.34 ± 0.26	3.44 ± 0.33	0.697
Inc. 3 800 ppm	-40.25 ± 0.21	-35.47 ± 0.55	4.60 ± 0.76	0.093

Instantaneous growth rates (μ<sub>i</sub>) are calculated from steady-state growth rates (Table 1), as described in Kukert and Riebesell (1998). Errors represent standard errors of means.

directly), to offset the latter enzyme's low CO<sub>2</sub> affinity and slow turnover rate. At low external CO<sub>2</sub> concentrations, an increase in both the CO<sub>2</sub> supply to Rubisco (i.e., CCM activity) and in the cellular concentrations of this enzyme appear to be necessary to maintain maximum rates of C fixation.

As a result of the inherent catalytic inefficiency of Rubisco, phytoplankton require high concentrations of this protein in the chloroplast. This enzyme is among the most abundant proteins in the cell, constituting a large fraction (up to ~25%) of cellular N pools (Ellis 1979). The modulation of Rubisco expression by CO<sub>2</sub> might, therefore, be expected to affect the overall biochemistry of the phytoplankton. Indeed, in a ~4.5-d incubation experiment (1998b in Table 1), a significant decrease in the ratios of C:N and carbohydrate:protein were observed at the lowest CO<sub>2</sub> treatment (100 ppm CO<sub>2</sub>), although no apparent differences existed between the intermediate (350 ppm) and high (800 ppm) CO<sub>2</sub> treatments. In incubation 1998c, no CO<sub>2</sub> effects on the biochemical composition of phytoplankton were detected (Fig. 9). Because these experiments lasted only ~2 d (part of which was required to equilibrate the CO<sub>2</sub> in bottles), it is possible that cells did not have sufficient time to adjust their bulk biochemical composition to the changes in CO<sub>2</sub>. In general, our results on CO<sub>2</sub>-dependent changes in C:N ratios are similar to those of Burkhardt et al. (1999b), who examined the effects of CO<sub>2</sub> variations on the elemental composition of several diatom species in laboratory cultures. A number of species showed significant decreases in C:N at CO<sub>2</sub> concentrations <350 ppm (~10 μM), with less-apparent changes at higher CO<sub>2</sub>. Other species showed no changes in C:N across all treatments (~100–800 ppm). More work will be needed to understand the potential effects of CO<sub>2</sub> on planktonic C:N ratios, given the important biogeochemical implications of this issue. Although recent studies have begun documenting significant deviations in planktonic C:N ratios from the canonical Redfield value of 6.6 (e.g., Sambrotto et al. 1993), the physiological mechanisms underlying this variability remain unknown. Higher phytoplankton C:N ratios would increase the magnitude of C export production in N-limited oceanic waters and could thus provide an important negative feedback to future atmospheric CO<sub>2</sub> increases.

**Stable C isotope fractionation**—Theoretical models show that C isotope fractionation by phytoplankton should depend on growth rates and external CO<sub>2</sub> concentrations for cells that rely on diffusive CO<sub>2</sub> uptake. More specifically, a plot of  $\epsilon_p$  (biological fractionation factor) versus growth rate ( $\mu$ )/[CO<sub>2</sub>] is expected to yield a straight line of negative (cell size-dependent) slope whose y-intercept equals the intrinsic fractionation factor of Rubisco— $\epsilon_r$  (Rau et al. 1997; Popp et al. 1998). A number of laboratory and field data sets exhibiting such behavior have indeed been reported (Bidigare et al. 1997; Popp et al. 1998) prompting the suggestion that species-specific sedimentary organic compound  $\delta^{13}\text{C}$  values may provide information on paleo CO<sub>2</sub> concentrations and/or phytoplankton growth rates in the oceans (Jasper and Hayes 1990). In contrast, the coastal diatom assemblages in our incubation experiments showed CO<sub>2</sub> and  $\mu$ -independent C isotope fractionation. Linear regression of  $\epsilon_p$  against the

ratio of growth rates to CO<sub>2</sub> concentrations ( $\mu/\text{CO}_2$ ) yielded a slope that was not significantly different from 0 ( $P > 0.1$ ; data not shown). The y-intercept of the regression line was  $7.10 \pm 0.63$  (SE), a value ~20‰ lower than the expected  $\epsilon_r$  of Rubisco. The expression of a form II Rubisco by the phytoplankton could partly explain the 20‰ discrepancy (Robinson and Cavanaugh 1995); however, the form II enzyme could not be detected in any of our field samples (C. Cavanaugh pers. comm). The lack of correlation between  $\epsilon_p$  and  $\mu/\text{CO}_2$  as well as the low y-intercept value cannot be reconciled with the purely diffusive CO<sub>2</sub> transport model. The isotope data thus provide clear evidence for active C uptake by the phytoplankton in our incubation experiments.

A number of recent laboratory and field studies have reported similar  $\mu/\text{CO}_2$ -insensitive, low fractionation factors. Values of  $\epsilon_p$  approaching 0‰ have been observed in marine and freshwater phytoplankton populations (e.g., Fry and Wainright 1991; Pancost et al. 1997, 1999; Kukert and Riebesell 1998; Burkhardt et al. 1999a; Rau et al., in press, F. Berman et al. pers. comm.). Isotope models and experimental data have demonstrated that the induction of a CCM decreases cellular  $\epsilon_p$  (Sharkey and Berry 1985; Goericke et al. 1994; Laws et al. 1997; Keller and Morel 1999). This effect has been attributed to a decrease in the rate of CO<sub>2</sub> leakage from cells relative to C fixation (Goericke et al. 1994). In addition, HCO<sub>3</sub><sup>-</sup> transport into cells followed by localized dehydration and rapid (e.g., CA-mediated) or quantitative utilization of the resulting CO<sub>2</sub> without isotopic equilibration between intracellular HCO<sub>3</sub><sup>-</sup> and CO<sub>2</sub> would result in significant <sup>13</sup>C enrichment of organic matter produced (i.e., lower fractionation). This is because the  $\delta^{13}\text{C}$  of this CO<sub>2</sub> would be as much as ~8‰–12‰ higher than that of CO<sub>2</sub> isotopically equilibrated with HCO<sub>3</sub><sup>-</sup> (Mook et al. 1974). Note that the same effect may be relevant in cells that take up CO<sub>2</sub> produced from the extracellular dehydration of HCO<sub>3</sub><sup>-</sup> via periplasmic CA, if the CO<sub>2</sub> taken up is not isotopically equilibrated with the ambient HCO<sub>3</sub><sup>-</sup>.

Although the  $\delta^{13}\text{C}$  signatures of the coastal phytoplankton assemblages strongly suggest that the cells possess a CCM, the isotope data per se cannot be used to infer the exact physiological mechanisms of inorganic C transport and assimilation (see Keller and Morel 1999). The very low  $\epsilon_p$  values we observed are most likely attributable to low C leakage out of cells (as expected of the CCM) and possibly to direct HCO<sub>3</sub><sup>-</sup> utilization. A high degree of  $\beta$ -carboxylation reactions as observed in *T. weissflogii* (Reinfelder et al. in press; see above) could also contribute to the low isotopic fractionation of the coastal phytoplankton, since PEP-carboxylase fixes an isotopically heavy DIC substrate (HCO<sub>3</sub><sup>-</sup>) with a low intrinsic fractionation factor (1‰–3‰; O'Leary 1981). Previous laboratory and field studies have suggested that various  $\beta$ -carboxylases may play a role in photosynthetic C assimilation and fractionation in phytoplankton (Descolas-Gros and Fontugne 1985, 1990). The coastal phytoplankton we sampled did not show detectable levels of PEP-C activity relative to a *T. weissflogii* positive control (significant activity may have been lost in frozen field samples; J. Reinfelder pers. comm.) but may have used an alternative C<sub>4</sub> pathway based on PEP-carboxylase (PEP-CK; see Descolas-Gros and Fontugne 1985). The PEP-

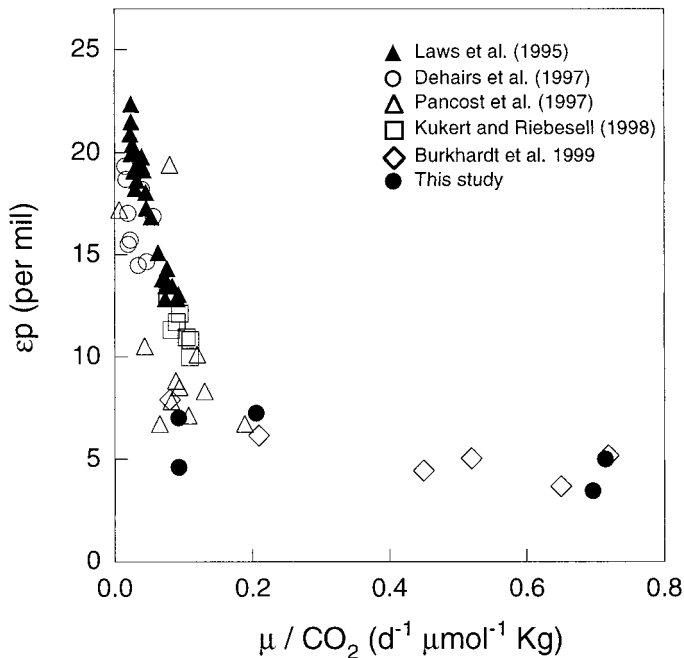


Fig. 10. Compilation of field and laboratory data of stable C isotope fractionation, phytoplankton growth rates, and CO<sub>2</sub> concentrations. Literature data were collected from the sources listed on the figure. Instantaneous growth rates were calculated as described by Kukert and Riebesell (1998).

CK C<sub>4</sub> pathway has a potentially different isotope effect than the PEP-C system, given that former enzyme uses CO<sub>2</sub> as a substrate and has an intrinsic fractionation factor (~25‰) that is similar to that of Rubisco (*see* Goericke et al. 1994 and references therein).

The presence of active C uptake mechanisms potentially complicates the interpretation and modeling of oceanic δ<sup>13</sup>C variations. A partial compilation of field and laboratory data (Fig. 10) shows that the diffusion-based model of C isotope fractionation (linear ε<sub>p</sub> vs. μ/CO<sub>2</sub>) cannot account for all observations (neglecting potential isotopic differences among studies imparted by variations in cell surface area/volume ratios; Rau et al. 1997; Popp et al. 1998). Significant curvature in the relationship is apparent at μ/CO<sub>2</sub> values >0.2, where active C uptake presumably contributes significantly to cellular C acquisition. Even at lower μ/CO<sub>2</sub> values where the ε<sub>p</sub> versus μ/CO<sub>2</sub> relationship appears to be linear, active C uptake by cells cannot be ruled out (Keller and Morel 1999). (Note that instantaneous growth rate estimates [μ<sub>i</sub>] were used to calculate μ/CO<sub>2</sub>; μ<sub>i</sub> values are higher than daily averaged growth rates, since they assume that all C uptake occurs in the light and correct for dark respiration; *see* Kukert and Riebesell 1998.) A μ/CO<sub>2</sub> value of 0.2 would occur for phytoplankton growing at μ<sub>i</sub> > 2 d<sup>-1</sup> (μ<sub>24hr</sub> > 1.1 d<sup>-1</sup> for 15 h of daylight) in air-equilibrated seawater with ~10 μM CO<sub>2</sub>. Such growth rates are not uncommon for natural phytoplankton communities (e.g., Furnas 1991; Jones et al. 1996; Strom and Strom 1996). During periods of rapid phytoplankton growth and biomass accumulation, μ/CO<sub>2</sub> values may greatly exceed 0.2 as dissolved CO<sub>2</sub> concentrations fall to levels as low as 3 μM (Codispoti et al. 1982). Similarly,

during glacial periods, aqueous CO<sub>2</sub> concentrations at equilibrium with the atmosphere were significantly lower than present-day values, and μ/CO<sub>2</sub> would thus have been higher for a given growth rate. Several authors have suggested that productivity (and presumably phytoplankton growth rates) in certain oceanic high nutrients low chlorophyll regions increased during glacial periods, possibly in response to elevated aeolian Fe deposition (e.g., Martin 1990; Kumar et al. 1995). Therefore, as a result of elevated growth rates and/or lower CO<sub>2</sub>, it seems likely that phytoplankton in glacial oceans experienced significantly greater μ/CO<sub>2</sub> values (for a given cell size), requiring even more CCM activity than is evident in the present-day ocean. The above discussion serves to illustrate that isotope models more complex than those based on passive CO<sub>2</sub> diffusion are required for understanding the wide variations in isotope composition encountered in nature.

**CO<sub>2</sub> and phytoplankton growth**—Over the past decade, CO<sub>2</sub> limitation of phytoplankton growth has been a topic of increasing interest among oceanographers. Despite this, very few data exist on the effects of CO<sub>2</sub> variations on marine primary productivity. Although laboratory studies and the results of a few short-term field experiments have been used to predict the responses of phytoplankton to CO<sub>2</sub> perturbations, almost no information is available on the steady-state (>1 generation time) effects of CO<sub>2</sub> on the growth rates of natural marine phytoplankton assemblages.

We have previously published the results of our first long-term CO<sub>2</sub> controlled incubation (Tortell et al. 1997), in which phytoplankton growth rates were constant at 100, 350, and 750 ppm CO<sub>2</sub> (~4, 14, and 30 μM CO<sub>2</sub>). This incubation was conducted in highly productive coastal waters dominated by large, fast-growing diatoms that significantly deplete CO<sub>2</sub> from the surface waters. Riebesell et al. (1993) suggested that primary production in such oceanic regions should be most susceptible to CO<sub>2</sub> limitation if the phytoplankton rely solely on diffusive CO<sub>2</sub> uptake. The ability of the diatoms to maintain maximum growth rates over a wide range of CO<sub>2</sub> concentrations provides evidence that they employ some form of C concentrating mechanism.

The results of five additional coastal CO<sub>2</sub> manipulation experiments (Table 1) strongly support our earlier conclusions (Tortell et al. 1997). In all but one case, phytoplankton growth rates were not significantly affected by the CO<sub>2</sub> changes. (Note, however, that differences in growth rates on the order of <10% could not be detected given the standard error of our growth-rate estimates.) The one instance in which we found a significant CO<sub>2</sub> effect was in a particularly fast-growing assemblage (μ > 2 d<sup>-1</sup>) whose growth was reduced by ~30% at 100 ppm (~4 μM) CO<sub>2</sub> relative to the 350 and 800 ppm (~14 and 32 μM) CO<sub>2</sub> treatments. This result suggests that phytoplankton may be unable to meet the high C demands imposed by very rapid growth rates at aqueous CO<sub>2</sub> concentrations <5 μM. Such low CO<sub>2</sub> concentrations have been observed in highly productive coastal waters (Codispoti et al. 1982) and would have been common in glacial times, at least in low-latitude waters in equilibrium with an atmospheric pCO<sub>2</sub> of ~180 ppm (Rau et al. 1991). It is also possible, however, that the assemblage was not able to fully acclimate to low CO<sub>2</sub> during the relatively short

incubation (~2 d total; ~1 d with equilibrated CO<sub>2</sub> in bottles) and that steady-state CO<sub>2</sub> limitation would not have been observed in a longer experiment.

In general, the responses of marine phytoplankton to CO<sub>2</sub> changes may be time-dependent. Two previous field studies in the Atlantic Ocean have examined the effects of CO<sub>2</sub> manipulations on productivity in short-term (2–6 h) experiments (Chen and Durbin 1994; Hein and Sand Jensen 1997). Both studies—one conducted in coastal waters and the other in the open ocean—report a ~25% decrease in photosynthetic <sup>14</sup>C uptake in response to a threefold reduction in CO<sub>2</sub> (from ~10 to 3 μM). Our short-term kinetic measurements (Fig. 3b) suggest that a similar decrease in C fixation would occur in Pacific phytoplankton assemblages acclimated to air levels of CO<sub>2</sub>. Photosynthetic rates, calculated by use of the Michaelis-Menten equation, are >95% of V<sub>max</sub> at 10 μM CO<sub>2</sub> (air-equilibrated concentrations) but reduced by 28% at 3 μM CO<sub>2</sub>. However, our incubation results indicate that such instantaneous CO<sub>2</sub> limitation is not indicative of a steady-state response, since phytoplankton adapt physiologically to low CO<sub>2</sub> by upregulating the CCM and increasing their C affinity over timescales of hours to days (Figs. 7, 8). As a result, long-term and short-term studies of phytoplankton CO<sub>2</sub> limitation are likely to produce different results.

Although the phytoplankton assemblages we studied showed little evidence for growth-rate limitation over a wide range of CO<sub>2</sub> concentrations, our results do not necessarily imply that changes in oceanic CO<sub>2</sub>—either anthropogenic or over glacial-interglacial cycles—should have no effects on coastal phytoplankton communities or primary productivity in open ocean regimes. The expression of a CO<sub>2</sub>-dependent CCM indicates that, from a purely biochemical standpoint, cells are inherently CO<sub>2</sub> stressed. This is not surprising, given the poor affinity of Rubisco for CO<sub>2</sub> and the low concentrations of this DIC species in seawater. Because phytoplankton must expend considerable resources (light, nitrogen, and Zn/Co/Cd) to acquire inorganic C, variations in ambient CO<sub>2</sub> levels that affect the mechanisms of inorganic C acquisition may affect the efficiency with which cells use these potentially limiting resources (Raven and Johnston 1991). Morel et al. (1994) provide an example of such a nutrient–C interaction. They showed that Zn deficiency in *T. weissflogii* inhibited HCO<sub>3</sub><sup>-</sup> utilization (by reducing CA activity), resulting in CO<sub>2</sub> limitation of cells. In addition, several laboratory and field studies have demonstrated that phytoplankton grown under low CO<sub>2</sub> conditions have significantly higher (1.4–2-fold) photosynthetic quantum requirements (Badger and Andrews 1982; Bürger et al. 1988; Berman-Frank et al. 1998). The data presented in this paper and that of Burkhardt et al. (1999b) suggest that CO<sub>2</sub> availability may influence cellular N demands. Under the nutrient- and light-replete conditions of our incubation experiments, changes in resource use efficiencies associated with the induction and repression of CCMs may not significantly alter the overall photosynthetic performance of cells. In offshore oligotrophic environments or low light conditions, however, limitations of one or more nutrients could constrain inorganic C acquisition and affect cellular responses to CO<sub>2</sub> changes.

Although our 2–5 d incubation experiments enabled us to study the physiological responses of indigenous phytoplank-

ton to CO<sub>2</sub> manipulations, they provided little insight into the potential, long-term ecological effects of CO<sub>2</sub> on phytoplankton communities. Raven (1990) has argued that phytoplankton that possess CCMs should respond differently to CO<sub>2</sub> variations than those species that rely on CO<sub>2</sub> diffusion. More generally, changing CO<sub>2</sub> levels may have differential effects on phytoplankton groups (diatoms, dinoflagellates, coccolithophores, and cyanobacteria) that utilize distinct mechanisms of C acquisition. Significant taxonomic variability exists among phytoplankton in the kinetic properties of Rubisco and in the capacity of cells to accumulate internal inorganic C pools (Badger et al. 1998; Tortell 2000). Taxa that possess catalytically efficient forms of Rubisco (high affinity for CO<sub>2</sub>) appear to rely less on C concentrating mechanisms to achieve maximum photosynthetic rates. For example, cellular C concentrating factors measured in diatoms (*K<sub>m</sub>* Rubisco ~30–60 μM) are significantly lower than those reported for cyanobacteria (*K<sub>m</sub>* Rubisco ~100–200 μM). This biochemical variation should affect the resource costs associated with C assimilation among taxa (Raven and Johnston 1991). As a result, the physiological response of diatom-dominated plankton communities to CO<sub>2</sub> manipulations may differ substantially from that of oligotrophic picoplankton populations.

Over the course of our incubations, we did not observe significant CO<sub>2</sub> effects on the species composition of coastal phytoplankton assemblages. This is perhaps not surprising, given the rather short duration of the experiments (2–5 d). In HNLC regions, iron additions induce dramatic changes in phytoplankton community composition on the timescale of several days to a week (Cavender-Bares et al. 1999). Compared with the strong differential effects of Fe limitation on various phytoplankton groups, competitive differences among taxa for inorganic C uptake may be relatively small, so that potential CO<sub>2</sub>-dependent shifts in species composition would occur over correspondingly longer timescales. Such slow ecosystem changes—although of great potential importance—may be very difficult to detect in typical field studies. Long-term monitoring programs, as well as laboratory competition experiments aimed at demonstrating CO<sub>2</sub>-dependent shifts in the competitive hierarchies among phytoplankton, may be an indirect way to address this question.

*Conclusions and future prospects*—The coastal phytoplankton assemblages we studied employed CO<sub>2</sub>-regulated mechanisms of active C uptake and showed little evidence of growth-rate limitation over a wide range of CO<sub>2</sub> concentrations (100–800 ppm). Although our results provide important information on the effects CO<sub>2</sub> on the physiology and growth of natural phytoplankton populations, much more research will be needed to understand how marine primary producers will respond both qualitatively and quantitatively to future anthropogenic CO<sub>2</sub> perturbations. Of particular importance is an understanding of potential biogeochemical feedbacks related to changes in the efficiency of the biological C pump—e.g., CO<sub>2</sub>-dependent changes in planktonic C:N ratios. Further information on the mechanisms of C acquisition by phytoplankton in situ (e.g., the role of trace metals such as Zn and the importance of β-carboxylases) will facilitate more robust interpretations of <sup>13</sup>C/<sup>12</sup>C variations in present-day phytoplankton communities

and in their sedimentary remains. Future work on C limitation of marine primary productivity may need to consider the possibility of CO<sub>2</sub>-dependent taxonomic shifts and the possibility of colimitations between C and other major and micronutrients.

## References

- BADGER, M. R., AND T. J. ANDREWS. 1982. Photosynthesis and inorganic carbon usage by the marine cyanobacterium *Synechococcus sp.* *Plant Physiol.* **70**: 517–523.
- , ———, S. M. WHITNEY, M. LUDWIG, D. C. YELLOWLEES, W. LEGGAT, AND G. D. PRICE. 1998. The diversity and coevolution of Rubisco, plastids, pyrenoids, and chloroplast based CO<sub>2</sub>-concentrating mechanisms in algae. *Can. J. Bot.* **76**: 1052–1071.
- , A. KAPLAN, AND J. A. BERRY. 1980. Internal inorganic carbon pool of *Chlamydomonas-Reinhardtii*—evidence for a carbon-dioxide concentrating mechanism. *Plant Physiol.* **66**: 407–413.
- BERMAN-FRANK, I., J. EREZ, AND A. KAPLAN. 1998. Changes in inorganic carbon uptake during the progression of a dinoflagellate bloom in a lake ecosystem. *Can. J. Bot.* **76**: 1043–1051.
- , T. ZOHARY, J. EREZ, AND Z. DUBINSKY. 1994. CO<sub>2</sub> availability, carbonic anhydrase and the annual dinoflagellate bloom in Lake Kinneret. *Limnol. Oceanogr.* **39**: 1822–1834.
- BIDIGARE, R. R., AND OTHERS. 1997. Consistent fractionation of <sup>13</sup>C in nature and in the laboratory: Growth-rate effects in some haptophyte algae. *Global Biogeochem. Cycles* **11**: 279–292.
- BÜRGER, J., S. MIYACHI, P. GALLAND, AND H. SENGER. 1988. Quantum requirements of photosynthetic oxygen evolution and 77K fluorescence emission spectra in unicellular green algae grown under low and high CO<sub>2</sub> conditions. *Bot. Acta.* **101**: 229–232.
- BURKHARDT, S., U. RIEBESELL, AND I. ZONDERVAN. 1999a. Effects of growth rate, CO<sub>2</sub> concentration, and cell size on the stable carbon isotope fractionation in marine phytoplankton. *Geochim. Cosmochim. Acta* **63**: 3729–3741.
- , I. ZONDERVAN, AND RIEBESELL U. 1999b. Effect of CO<sub>2</sub> concentration on C:N:P ratio in marine phytoplankton: A species comparison. *Limnol. Oceanogr.* **44**: 683–690.
- CANVIN, D. T. 1990. Photorespiration and CO<sub>2</sub> concentrating mechanisms, p 253–273. *In* D. T. Dennis and D. H. Turpin. [eds.], *Plant Physiology, biochemistry and molecular biology*. Longman Scientific and Technical.
- CAVENDER-BARES, K. K., E. L. MANN, S. W. CHISHOLM, M. E. ONDRUSEK, AND R. R. BIDIGARE. 1999. Differential response of equatorial Pacific phytoplankton to iron fertilization. *Limnol. Oceanogr.* **44**: 237–246.
- CHEN, C. Y., AND E. G. DURBIN. 1994. Effects of pH on the growth and carbon uptake of marine phytoplankton. *Mar. Ecol. Progr. Ser.* **109**: 83–94.
- CODISPOTI, L. A., G. E. FRIEDERICH, R. L. IVERSON, AND D. W. HOOD. 1982. Temporal changes in the inorganic carbon system of the southeastern Bering Sea during spring 1980. *Nature* **296**: 242–245.
- COLEMAN, J. E. 1998. Zinc enzymes. *Curr. Opin. Chem. Biol.* **2**: 222–234.
- COLMAN, B., AND C. ROTATORE. 1988. Uptake and accumulation of inorganic carbon by a fresh-water diatom. *J. Exp. Bot.* **39**: 1025–1032.
- , AND ———. 1995. Photosynthetic inorganic carbon uptake and accumulation in 2 marine diatoms. *Plant Cell Environ.* **18**: 919–924.
- DEHAIRS, F., E. KOPCZYNSKA, P. NIELSEN, C. LANCELOT, D. C. E. BAKKER, W. KOEVE, AND L. GOEYENS. 1997. δC-13 of Southern Ocean suspended organic matter during spring and early summer: Regional and temporal variability. *Deep Sea Res. II* **44**: 129–142.
- DESCOLAS-GROS, C., AND M. FONTUGNE. 1985. Carbon fixation in marine phytoplankton—carboxylase activities and stable carbon-isotope ratios—physiological and paleoclimatological aspects. *Mar. Biol.* **87**: 1–6.
- , AND ———. 1990. Stable carbon isotope fractionation by marine phytoplankton during photosynthesis. *Plant Cell Environ.* **13**: 207–218.
- ELLIS, R. J. 1979. The most abundant protein in the world. *Trends Biochem. Sci.* **4**: 241–244.
- FALKOWSKI, P. G., A. SUKENIK, AND R. HERZIG. 1989. Nitrogen limitation of *Isochrysis galbana* (Haptophyceae) 2. Relative abundance of chloroplast proteins. *J. Phycol.* **25**: 471–478.
- FRY, B. 1996. <sup>13</sup>C/<sup>12</sup>C fractionation by marine diatoms. *Mar. Ecol. Progr. Ser.* **134**: 283–294.
- , AND S. W. WAINRIGHT. 1991. Diatom sources of <sup>13</sup>C-rich carbon in marine food webs. *Mar. Ecol. Progr. Ser.* **76**: 149–157.
- FURNAS, M. J. 1991. Net in situ growth rates of phytoplankton in an oligotrophic, tropical shelf ecosystem. *Limnol. Oceanogr.* **36**: 13–29.
- GOERICKE, R., J. P. MONTOYA, AND B. FRY. 1994. Physiology of isotopic fractionation in algae and cyanobacteria, p 187–221. *In* K. Lajtha and B. Michener [eds.], *Stable isotopes in ecology and environmental science*. Blackwell.
- HEIN, M., AND K. SAND-JENSEN. 1997. CO<sub>2</sub> increases oceanic primary production. *Nature* **388**: 526–527.
- HUTCHINS, D. A., AND K. W. BRULAND. 1998. Iron-limited diatom growth and Si: N uptake ratios in a coastal upwelling regime. *Nature* **393**: 561–564.
- JASPER, J. P., AND J. M. HAYES. 1990. A carbon isotope record of CO<sub>2</sub> levels during the late Quaternary. *Nature* **347**: 462–464.
- , ———, A. C. MISO, AND F. G. PRAHL. 1994. Photosynthetic fractionation of <sup>13</sup>C and concentrations of dissolved CO<sub>2</sub> in the central equatorial Pacific during the last 225,000 years. *Paleoceanography* **9**: 781–798.
- JOHNSTON, A. M., AND J. A. RAVEN. 1997. Inorganic carbon accumulation by the marine diatom *Phaeodactylum tricorutum*. *Eur. J. Phycol.* **31**: 285–290.
- JONES, D. R., D. M. KARL, AND E. A. LAWS. 1996. Growth rates and production of heterotrophic bacteria and phytoplankton in the North Pacific subtropical gyre. *Deep-Sea Res. Part I Oceanogr. Res. Pap.* **43**: 1567–1580.
- KAPLAN, A., AND L. REINHOLD. 1999. CO<sub>2</sub> concentrating mechanism in photosynthetic microorganisms. *Ann. Rev. Plant Phys. Plant Mol. Biol.* **50**: 539–570.
- KELLER, K., AND F. M. M. MOREL. 1999. A model of carbon isotopic fractionation and active carbon uptake in phytoplankton. *Mar. Ecol. Progr. Ser.* **182**: 295–298.
- KUKERT, H., AND U. RIEBESELL. 1998. Phytoplankton carbon isotope fractionation during a diatom spring bloom in a Norwegian fjord. *Mar. Ecol. Progr. Ser.* **173**: 127–137.
- KUMAR, N., R. F. ANDERSON, R. A. MORTLOCK, P. N. FROELICH, P. KUBIK, B. DITTRICHANNEN, AND M. SUTER. 1995. Increased biological productivity and export production in the glacial Southern Ocean. *Nature* **378**: 675–680.
- LALLI, C. M., AND T. R. PARSONS. 1994. *Biological oceanography: An introduction*. Pergamon Press.
- LANE, T. W., AND F. M. M. MOREL. 2000a. Regulation of carbonic anhydrase expression by zinc, cobalt, and carbon dioxide in the marine diatom *Thalassiosira weissflogii*. *Plant Physiol.* **123**: 1–8.
- , AND ———. 2000b. A biological function for cadmium in marine diatoms. *Proc. Nat. Acad. Sci.* **97**: 4627–4631.

- LAWSON, E. A., B. N. POPP, AND R. R. BIDIGARE. 1997. Effect of growth rate and CO<sub>2</sub> concentration on carbon isotopic fractionation by the marine diatom *Phaeodactylum tricorutum*. *Limnol. Oceanogr.* **42**: 1552–1560.
- , ———, ———, M. C. KENNICUTT, AND S. A. MACKO. 1995. Dependence of phytoplankton carbon isotopic composition on growth-rate and [CO<sub>2</sub>]<sub>(aq)</sub>—theoretical considerations and experimental results. *Geochim. Cosmochim. Acta* **59**: 1131–1138.
- MARTIN, J. H. 1990. Glacial—interglacial CO<sub>2</sub> change: The iron hypothesis. *Paleoceanography* **5**: 1–13.
- MILLERO, F. J. 1996. *Chemical oceanography*. CRC.
- MITCHELL, C., AND J. BEARDALL. 1996. Inorganic carbon uptake by an Antarctic sea-ice diatom, *Nitzschia frigida*. *Polar Biol.* **16**: 95–99.
- MOOK, W. G., J. C. BOMMERSON, AND W. H. STAVERMAN. 1974. Carbon isotope fractionation between dissolved bicarbonate and gaseous carbon dioxide. *Earth Planet. Sci. Lett.* **22**: 169–176.
- MOREL, F. M. M., J. R. REINFELDER, S. B. ROBERTS, C. P. CHAMBERLAIN, J. G. LEE, AND D. YEE. 1994. Zinc and carbon co-limitation of marine phytoplankton. *Nature* **360**: 740–742.
- MORONEY, J. V., AND Z. Y. CHEN. 1998. The role of the chloroplast in inorganic carbon uptake by eukaryotic algae. *Can. J. Bot.* **76**: 1025–1034.
- MORRIS, I. 1980. Paths of carbon assimilation in marine phytoplankton, p 139–160. *In* P. G. Falkowski [ed.], *Primary productivity in the sea*. C acquisition. Plenum.
- O'LEARY, M. H. 1981. Carbon isotope fractionation in plants. *Phytochemistry (Oxf)* **20**: 553–567.
- PANCOST, R. D., K. H. FREEMAN, AND S. G. WAKEHAM. 1999. Controls on the carbon-isotope compositions of compounds in Peru surface waters. *Org. Geochem.* **30**: 319–340.
- , ———, ———, AND C. Y. ROBERTSON. 1997. Controls on carbon isotope fractionation by diatoms in the Peru upwelling region. *Geochim. Cosmochim. Acta* **61**: 4983–4991.
- PARSONS, T. R., Y. MAITA, AND C. M. LALLI. 1984. *A manual of chemical and biological methods for seawater analysis*. Pergamon.
- POPP, B. N., E. A. LAWS, R. R. BIDIGARE, J. E. DORE, K. L. HANSON, AND S. G. WAKEHAM. 1998. Effect of phytoplankton cell geometry on carbon isotopic fractionation. *Geochim. Cosmochim. Acta* **62**: 69–77.
- RAU, G. H., F. P. CHAVEZ, AND G. E. FRIEDERICH. Plankton <sup>13</sup>C/<sup>12</sup>C Variations in Monterey Bay, CA: Evidence of non-diffusive inorganic carbon uptake by phytoplankton in an upwelling environment. *Deep-Sea Res.* (in press).
- , P. N. FROELICH, T. TAKAHASHI, AND D. J. DES MARAIS. 1991. Does sedimentary δ<sup>13</sup>C record variations in Quaternary ocean [CO<sub>2</sub>(aq)]? *Paleoceanography* **6**: 335–347.
- , U. RIEBESELL, AND D. A. WOLF-GLADROW. 1997. CO<sub>2(aq)</sub>-dependent photosynthetic <sup>13</sup>C fractionation in the ocean: A model versus measurements. *Global Biogeochem. Cycles* **11**: 267–278.
- , J.-L. TEYSSIE, R. RASSOULZADEGAN, AND S. W. FOWLER. 1990. <sup>13</sup>C/<sup>12</sup>C and <sup>15</sup>N/<sup>14</sup>N variations among size-fractionated marine particles: Implications for their origin and trophic relationships. *Mar. Ecol. Prog. Ser.* **59**: 33–38.
- RAVEN, J. A. 1990. Implications of inorganic carbon utilization: ecology, evolution, and geochemistry. *Can. J. Bot.* **69**: 908–924.
- . 1997. Inorganic carbon acquisition by marine autotrophs. *Adv. Bot. Res.* **27**: 85–209.
- , AND A. M. JOHNSTON. 1991. Mechanisms of inorganic-carbon acquisition in marine phytoplankton and their implications for the use of other resources. *Limnol. Oceanogr.* **36**: 1701–1714.
- REINFELDER, J. R., A. M. KRAPIEL, AND F. M. M. MOREL. Unicellular C<sub>4</sub> photosynthesis in a marine diatom. *Nature* (in press).
- RIEBESELL, U., D. A. WOLF-GLADROW, AND V. SMETACEK. 1993. Carbon dioxide limitation of marine phytoplankton growth rates. *Nature* **361**: 249–251.
- ROBERTS, S. B., T. W. LANE, AND F. M. M. MOREL. 1997. Carbonic anhydrase in the marine diatom *Thalassiosira weissflogii* (Bacillariophyceae). *J. Phycol.* **33**: 845–850.
- ROBINSON, J. J., AND C. M. CAVANAUGH. 1995. Expression of form I and form II Rubisco in chemoautotrophic symbioses: Implications for the interpretation of stable carbon isotope values. *Limnol. Oceanogr.* **40**: 1496–1502.
- SAMBROOK, J., E. F. FRITSCH, AND T. MANIATIS. 1989. *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory.
- SAMBROTTO, R. N., AND OTHERS. 1993. Elevated consumption of carbon relative to nitrogen in the surface ocean. *Nature* **363**: 248–250.
- SARMIENTO, J. L., AND C. LEQUÉRE. 1996. Oceanic carbon dioxide uptake in a model of century-scale global warming. *Science* **274**: 1346–1350.
- SHARKEY, T. D., AND J. A. BERRY. 1985. Carbon isotope fractionation in algae as influenced by inducible CO<sub>2</sub> concentrating mechanisms, p. 381–401. *In* W. J. Lucas and J. A. Berry [eds.], *Inorganic carbon uptake by aquatic photosynthetic organisms*. Am. Soc. Plant Physiologists.
- STROM, S. L., AND M. W. STROM. 1996. Microplankton growth, grazing, and community structure in the northern Gulf of Mexico. *Mar. Ecol. Prog. Ser.* **130**: 229–240.
- SÜLTEMEYER, D. C. 1998. Carbonic anhydrase in eukaryotic algae: characterization, regulation and possible function during photosynthesis. *Can. J. Bot.* **76**: 962–972.
- TALLING, J. F. 1976. The depletion of carbon dioxide from lake water by phytoplankton. *J. Ecol.* **64**: 79–121.
- TORTELL, P. D. 2000. Evolutionary and ecological perspectives on carbon acquisition in phytoplankton. *Limnol. Oceanogr.* **45**: 744–750.
- , J. R. REINFELDER, AND F. M. M. MOREL. 1997. Active uptake of bicarbonate by diatoms. *Nature* **390**: 243–244.

Received: 11 April 2000

Accepted: 4 July 2000

Amended: 31 July 2000