

## Bicarbonate transport and extracellular carbonic anhydrase activity in Bering Sea phytoplankton assemblages: Results from isotope disequilibrium experiments

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### Abstract

We used a  $^{14}\text{C}$  isotope disequilibrium technique to provide quantitative estimates of both direct  $\text{HCO}_3^-$  transport and extracellular CA activity in Bering Sea phytoplankton assemblages. The method revealed that direct  $\text{HCO}_3^-$  transport was the dominant mechanism of inorganic C uptake in both coastal and open ocean waters, accounting for more than half of the total C flux to the phytoplankton. The relative importance of  $\text{HCO}_3^-$  transport was not related to phytoplankton biomass, productivity, or ambient  $\text{CO}_2$  concentrations at individual sampling stations. However,  $\text{HCO}_3^-$  transport and total inorganic C uptake rates decreased in response to elevated  $\text{CO}_2$  in direct manipulation experiments. Kinetic analysis of the  $^{14}\text{C}$  time-course data revealed low levels of extracellular carbonic anhydrase activity at most stations. This activity was related to phytoplankton taxonomic compositions and/or  $\text{CO}_2$  concentrations, but was relatively unaffected by direct  $\text{CO}_2$  manipulations.

In recent years, inorganic carbon acquisition by marine phytoplankton has attracted increasing interest among the oceanographic community. This stems from a need to understand potential  $\text{CO}_2$  effects on oceanic productivity (Riebesell 2004) and the controls on the C isotope signatures of marine organic matter (Goericke et al. 1994). Previous work suggested the possibility of  $\text{CO}_2$  limitation of phytoplankton growth in the oceans (Riebesell et al. 1993; Hein and Sand-Jensen 1997). However, numerous laboratory studies with a variety of phytoplankton species have documented the existence of carbon concentrating mechanisms (CCMs) through which cells accumulate high intracellular inorganic C concentrations to saturate C fixation by RubisCO—the rate-limiting carboxylase in the Calvin cycle (see Raven 1997; Kaplan and Reinhold 1999; Colman et al. 2002). Inorganic C uptake associated with the CCM can involve the active transport of  $\text{HCO}_3^-$  (the predominant form of inorganic C in seawater) and/or  $\text{CO}_2$ , as well as the presence of extracellular carbonic anhydrase (eCA). This enzyme catalyzes the otherwise slow interconversion between  $\text{HCO}_3^-$  and  $\text{CO}_2$  and, thus, functions as part of an indirect  $\text{HCO}_3^-$  uptake system whereby cells transport  $\text{CO}_2$  that is released from  $\text{HCO}_3^-$  dehydration (Sültemeyer et al. 1993; Badger 2003). Despite some common elements, the exact mechanisms of C uptake vary significantly among phytoplankton, with different species depending on  $\text{HCO}_3^-$

transport,  $\text{CO}_2$  transport, eCA activity or a combination (Colman et al. 2002).

While inorganic C acquisition has been relatively well characterized in a number of individual phytoplankton species grown in laboratory cultures, little information is available on the C uptake physiology of natural phytoplankton assemblages in the oceans. Recent work has made progress toward documenting the existence of CCMs in marine phytoplankton populations in situ (Tortell et al. 2000; Tortell and Morel 2002; Cassar et al. 2004). Tortell et al. (1997, 2000) demonstrated that phytoplankton in the oceans had the capacity to concentrate inorganic carbon and maintain rapid growth rates over a wide range of  $\text{CO}_2$  concentrations. This work suggested that  $\text{HCO}_3^-$  was an important source of inorganic C for phytoplankton and showed that CCM activity and cellular biochemistry were modulated by ambient  $\text{CO}_2$  concentrations. It did not, however, provide strict quantitative estimates for the relative proportions of  $\text{HCO}_3^-$  and  $\text{CO}_2$  taken up by cells. Two subsequent field studies (Tortell and Morel 2002; Cassar et al. 2004) have employed the isotope disequilibrium method (Espie and Colman 1986; Elzenga et al. 2000) to explicitly quantify the contribution of  $\text{HCO}_3^-$  to total C uptake by natural phytoplankton assemblages. The results obtained from these experiments showed that  $\text{HCO}_3^-$  contributes significantly to phytoplankton C uptake in both the equatorial Pacific and Southern Ocean and demonstrated that  $\text{HCO}_3^-$  use can be modulated by external  $\text{CO}_2$  concentrations.

The existing field studies of inorganic C acquisition have provided important information for phytoplankton in the Southern Ocean and subtropical/equatorial Pacific Ocean, but the extent to which the results apply to other oceanic regions is unclear. More data are needed from a variety of marine environments to gain a comprehensive understanding of how phytoplankton acquire inorganic carbon in

### Acknowledgments

We thank Ken Bruland and David Hutchins for providing berths on their research cruise and the scientists and crew of the R/V *Kilo Moana* for logistical assistance at sea. Funding for this research was provided by the Natural Sciences and Engineering Council of Canada, and from the U.S. National Science Foundation (grant OCE 032774 to D. Hutchins).

seawater. Here we present results of C uptake studies in the southeastern (SE) Bering Sea conducted using the isotope disequilibrium method. Previous field studies have documented the impact of biological activity on the carbonate system of this oceanic region, demonstrating extremely low surface water  $\rho\text{CO}_2$  (Codispoti et al. 1982) with sharp gradients over small spatial scales (Tortell 2005b). In contrast, no information is available on the physiological mechanisms of C acquisition by indigenous phytoplankton in these waters or the potential effects of  $\text{CO}_2$  variability on plankton physiology.

Our results show that  $\text{HCO}_3^-$  is the predominant C species taken up by phytoplankton communities in all regions of the SE Bering Sea and suggest that a large fraction of  $\text{HCO}_3^-$  uptake occurs through a direct transport mechanism. In addition,  $\text{CO}_2$  incubation experiments demonstrate that  $\text{HCO}_3^-$  use by phytoplankton can be regulated by ambient  $\text{CO}_2$  concentrations. Data obtained from similar experiments conducted in the Subarctic Pacific (Tortell et al. 2006) suggest that the results presented here may be broadly applicable to large regions of the coastal and open North Pacific Ocean.

## Materials and methods

Field experiments were conducted during August and September 2003 aboard the R/V *Kilo Moana*. Sampling sites (Fig. 1) were located across three regions of the southeastern Bering Sea: the open ocean domain (western gyre), the continental shelf break, and the continental shelf domain (see Loughlin and Ohtani 1999). Nutrient concentrations, chlorophyll *a* (Chl *a*),  $\text{CO}_2$  concentrations, primary productivity, and calcification rates were measured at most stations. Nutrient concentrations were determined with an onboard autoanalyzer, Chl *a* was measured by fluorometric analysis (Parsons et al. 1984), and  $\text{CO}_2$  partial pressure at 8 of the 11 stations was obtained from a membrane-inlet mass spectrometer (Tortell 2005a). For each station, phytoplankton community composition was qualitatively examined by microscopic examination of concentrated samples preserved with 2% (final concentration) formaldehyde. Samples were concentrated as described below for isotope disequilibrium experiments, and the relative dominance of nanoflagellates and diatoms was assessed. Rates of planktonic primary productivity and calcification were determined using short-term  $^{14}\text{C}$  uptake experiments. Triplicate sample bottles and one dark bottle containing 200 mL of seawater, collected from the Chl *a* maximum (10–30 m; determined by Chl *a* fluorescence profiles), were incubated for 8 h with  $^{14}\text{C}_i$  (1.3 GBq,  $\sim 6,600 \text{ MBq mol}^{-1}$ ), then gently filtered ( $<100 \text{ mm Hg}$  vacuum) onto 0.6-mm polycarbonate filters. The amounts of particulate organic and inorganic carbon production were determined using the protocol described by Lam et al. (2001). Incubations were conducted in a shipboard flow-through chamber maintained at ambient sea-surface temperature ( $\pm 1^\circ\text{C}$ ), with two layers of neutral-density screening to provide  $\sim 30\%$  of surface irradiance.

Phytoplankton samples used for C uptake experiments were collected from the Chl *a* maximum. Approximately

10–20 liters of seawater were gravity-filtered directly from Niskin bottles onto 2 or 20  $\mu\text{m}$ , 47-mm polycarbonate membranes. Phytoplankton collected on filters were immediately resuspended into  $\sim 10 \text{ mL}$  of pH 8.50 seawater (buffered with 20  $\text{mmol L}^{-1}$  bicine), and experiments begun within 15 min. The final concentration of chlorophyll in concentrated cell suspensions ranged from  $\sim 0.2$  to 4  $\text{mg L}^{-1}$ . To quantify the relative contributions of  $\text{CO}_2$  and  $\text{HCO}_3^-$  to C uptake by phytoplankton assemblages, we used the isotope disequilibrium technique (Espie and Colman 1986; Elzenga et al. 2000) following the protocol of Tortell and Morel (2002) with a few minor modifications. Briefly, 2.5 mL of concentrated cell suspensions in seawater buffer were acclimated for 5 min at  $10^\circ\text{C}$  in an oxygen electrode chamber illuminated with a slide projector at  $\sim 300 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ . To initiate the experiments, a 2.66 GBq  $^{14}\text{C}$  spike preequilibrated in 100  $\mu\text{L}$  of a pH 7.0, 50  $\text{mmol L}^{-1}$  Hepes buffer was injected into the concentrated cell suspension. After the injection of the  $^{14}\text{C}$  spike, subsamples of 200  $\mu\text{L}$  were withdrawn at various intervals, between 5 and 300 s, and dispensed into 1 mL of 50% HCl. Acidified samples were placed in a fume hood and degassed for 4 h by purging air into each sample vial. This degassing period allowed for removal of residual inorganic  $^{14}\text{C}$  that had not been incorporated into photosynthetic products. Experiments were conducted in the presence and absence of the membrane-impermeable CA inhibitor acetazolamide (AZ) to examine the role of extracellular CA in C uptake by phytoplankton assemblages. The inhibitor was added to cell suspensions at a final concentration of 20  $\mu\text{mol L}^{-1}$  at least 10 min before beginning experiments. All experimental treatments (i.e., control and +AZ) were conducted in duplicate, and all individual data points were used for the analysis.

*Incubation experiments*—Shipboard  $\text{CO}_2$ -manipulation experiments were conducted following the protocol of Tortell et al. (2002). Seawater was collected in the oceanic domain (high-nutrient, low-chlorophyll waters), and 5  $\text{nmol L}^{-1}$  iron was added to promote phytoplankton growth. Triplicate incubation bottles were continuously bubbled with 15.2 Pa (150 ppm) or 76 Pa (750 ppm)  $\text{CO}_2$  (balance air) supplied from commercially prepared gas mixtures (1 Pa = 9.87 ppm = 9.87  $\mu\text{atm}$ ). Nutrient concentrations, Chl *a*, species composition, primary productivity, and calcification rates were measured in incubation samples as described above for the in situ samples. Measurements of Chl *a* over time were used to derive growth rates for each  $\text{CO}_2$  incubation treatment. We carried out two separate  $\text{CO}_2$ -manipulation experiments, in which isotope disequilibrium experiments were performed for each  $\text{CO}_2$  treatment. Samples from each of the replicate incubation bottles were pooled together and filtered onto 2- $\mu\text{m}$  polycarbonate filters. Isotope disequilibrium experiments were performed as described above.

*Data analysis and modeling*—The isotope disequilibrium method was designed to quantify the relative contributions of  $\text{HCO}_3^-$  and  $\text{CO}_2$  to C uptake by phytoplankton during steady-state photosynthesis (Espie and Colman 1986;

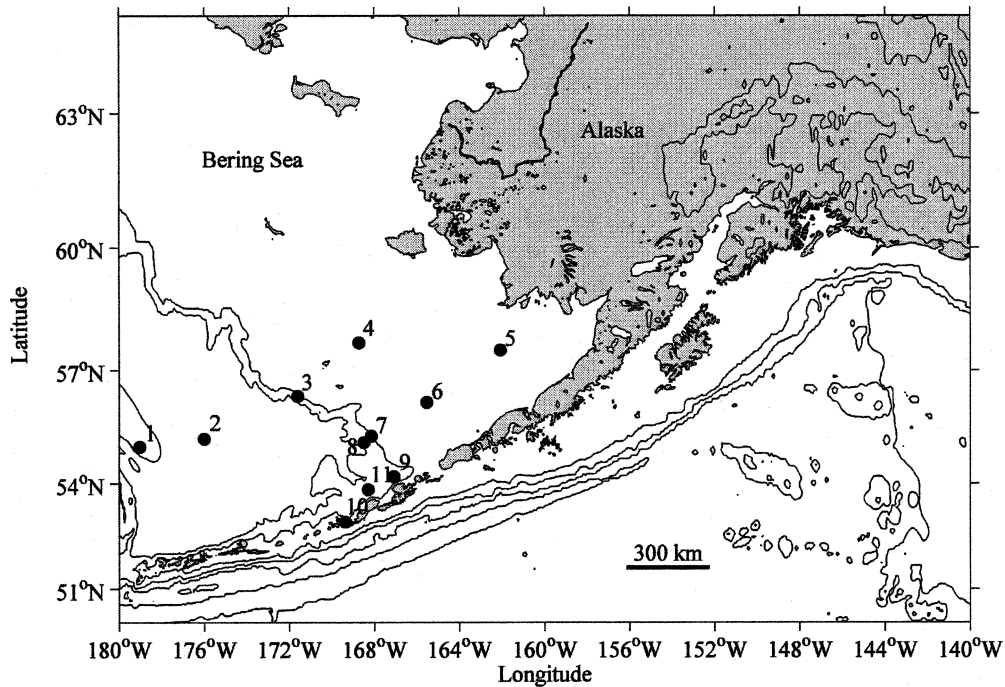


Fig. 1. Location of sampling sites in the SE Bering Sea.

Elzenga et al. 2000; Tortell and Morel 2002). The method is based upon the slow (uncatalyzed) interconversion between  $\text{HCO}_3^-$  and  $\text{CO}_2$  (Johnson 1982), which enables these chemical species to be differentially labeled with  $^{14}\text{C}$  over time periods of several minutes. In the initial  $^{14}\text{C}$  spike solution, at pH 7.0,  $^{14}\text{CO}_2$  accounts for  $\sim 20\%$  of the total dissolved inorganic carbon (DIC), whereas in the cell suspension, at pH 8.5, of the total DIC 0.3% exists as  $\text{CO}_2$  (Morel and Hering 1993). Following the addition of the  $^{14}\text{C}$  spike, the specific activity of  $\text{CO}_2$  in the seawater buffer is initially high, decaying exponentially to an equilibrium value over a period of several minutes. In contrast, the initial specific activity of  $^{14}\text{HCO}_3^-/^{14}\text{CO}_3^{2-}$  in the seawater buffer is close to its equilibrium value and, therefore, changes relatively little during the time course. (Note that the  $^{14}\text{C}$  tracer addition does not significantly affect the bulk concentration or chemical speciation of the unlabeled DIC in solution.)

The instantaneous rate of  $^{14}\text{C}$  uptake by phytoplankton (the sum of  $^{14}\text{CO}_2$  and  $\text{H}^{14}\text{CO}_3^-$  uptake rates) is affected by the changes in the specific activities of  $\text{CO}_2$  and  $\text{HCO}_3^-$  during the experimental time course. Thus, cells taking up only  $\text{CO}_2$  will show a significant decrease in their apparent  $^{14}\text{C}$  uptake rates, while  $^{14}\text{C}$  uptake rates will remain nearly constant for cells transporting only  $\text{HCO}_3^-$ . In practice, it is the accumulation of  $^{14}\text{C}$ , rather than the instantaneous uptake rate, which is measured in time-course experiments. As a result, the uptake curves are best modeled in their integral form (modified from Elzenga et al. 2000):

$$\text{DPM}_t = V_i(1-f)(\alpha_1 t + (\Delta\text{SA}_{\text{CO}_2}/\text{SA}_{\text{DIC}}) \times (1 - e^{-\alpha_1 t}))/\alpha_1 + V_i(f)(\alpha_2 t + (\Delta\text{SA}_{\text{HCO}_3^-}/\text{SA}_{\text{DIC}}) \times (1 - e^{-\alpha_2 t}))/\alpha_2 \quad (1)$$

$V_i$  is the total rate of C uptake;  $f$  is the fraction of uptake attributable to  $\text{HCO}_3^-$ ;  $\alpha_1$  and  $\alpha_2$  are the temperature, salinity, and pH-dependent first-order rate constants for  $\text{CO}_2$  and  $\text{HCO}_3^-$  hydration and dehydration, respectively;  $\Delta\text{SA}_{\text{CO}_2}$  and  $\Delta\text{SA}_{\text{HCO}_3^-}$  are the differences between the initial and equilibrium values of the specific activity of  $\text{CO}_2$  and  $\text{HCO}_3^-$ ; and  $\text{SA}_{\text{DIC}}$  is the specific activity of all inorganic carbon species at equilibrium. During steady-state photosynthesis,  $V_i$  and  $f$  are assumed to be constant so that changes in the instantaneous  $^{14}\text{C}$  uptake rate reflect only changes in the specific activity of the two C species. In the absence of eCA activity,  $\alpha_1$  and  $\alpha_2$  are 0.0152 and 0.0173  $\text{s}^{-1}$ , respectively, under our experimental conditions (temperature = 10°C, salinity = 34, pH values 8.5). These values were calculated from the equations of Espie and Colman (1986) using temperature and salinity corrections from Johnson (1982). The values of  $\Delta\text{SA}_{\text{CO}_2}/\text{SA}_{\text{DIC}}$  and  $\Delta\text{SA}_{\text{HCO}_3^-}/\text{SA}_{\text{DIC}}$  are set by the difference in pH between the  $^{14}\text{C}$  spike and seawater buffer, with the values of 49 and  $-0.24$ , respectively. These values are not affected by the presence of carbonic anhydrase.

Previous authors have used various numerical approaches to quantify the proportions of  $\text{HCO}_3^-$  and  $\text{CO}_2$  taken up in isotope disequilibrium experiments. Elzenga et al. (2000) fit data with the integral equation (Eq. 1), while Tortell and Morel (2002) and Cassar et al. (2004) derived the ratio of initial and final  $^{14}\text{C}$  uptake rates. To account for potential eCA activity, experiments were run in the presence of membrane-impermeable CA inhibitors (AZ or DBS), and the rate constant,  $\alpha$ , was presumed to equal its uncatalyzed value. While these methods produce estimates of  $\text{HCO}_3^-$  and  $\text{CO}_2$  contributions to total C uptake, the uncertainty associated with these parameters has not been

addressed explicitly in previous studies. (We have found that there can be large parameter errors even when the overall curve fits appear to be excellent.) Moreover, the analysis of initial and final uptake rates (Tortell and Morel 2002; Cassar et al. 2004) does not provide any means to quantify eCA activity. Finally, the use of AZ treatments requires the assumption that the inhibitor has no direct effects on the C uptake system.

In the analysis presented here, we used the integrated form of the uptake Eq. 1 to quantify both the fraction of  $\text{HCO}_3^-$  used and the extent of eCA expression. We fit this equation to our  $^{14}\text{C}$  time-course data using the Marquand-Levenberg nonlinear regression algorithm (SigmaPlot) and obtained best fit parameter estimates and standard errors for  $V_i$  and  $f$ . In samples treated with AZ, uncatalyzed rate constants were used for  $\alpha_1$  and  $\alpha_2$ . For control experiments, eCA activity was estimated from the data fits by allowing the rate constant,  $\alpha_1$ , to vary as a model parameter, constrained to be equal to or greater than the uncatalyzed value. (Values of  $\alpha_2$  can be calculated directly from  $\alpha_1$ .) In this manner, eCA expression was modeled as an increase in the rate of  $\text{CO}_2/\text{HCO}_3^-$  equilibration (hereafter referred to as  $\alpha$ ). With this formulation, the parameter  $f$  represents the contribution of direct  $\text{HCO}_3^-$  transport, while indirect  $\text{HCO}_3^-$  use is represented as  $\text{CO}_2$  uptake coupled with increased  $\alpha$ .

To test the robustness of our quantitative analysis, we applied the nonlinear fitting procedure to a series of hypothetical  $^{14}\text{C}$  time-course data with specified values of  $f$  and  $\alpha$ . A random error of up to 15% was introduced to simulate experimental noise in the test data sets. Across a large range of  $f$  and  $\alpha$  values, we found excellent agreement between the expected and model-derived parameter values (Fig. 2). The mean deviation between expected and predicted parameters was  $<5\%$  for  $\alpha$ , and  $<8\%$  for  $f$ . There was, however, a tendency for the model to overestimate  $f$  at low values, and the relative error in the estimation of this parameter increased as the parameter decreased. Overall, for  $f$  values greater than  $\sim 25\%$ , the curve-fitting algorithm appeared capable of providing accurate estimates of  $f$  and  $\alpha$  when both of these parameters were allowed to vary simultaneously.

## Results

*In situ stations*—Sampling sites in the offshore region (western gyre), shelf break, and continental shelf domains differed significantly in their chemical and biological properties. Nutrient concentrations, phytoplankton biomass (Chl *a*),  $\text{CO}_2$  concentrations, primary productivity, and qualitative phytoplankton taxonomic composition of the stations are given in Table 1. In the western gyre (Stas. 1 and 2), nutrient concentrations were generally high ( $>10 \mu\text{mol L}^{-1}$ ), while Chl *a* values were typically low ( $<1 \mu\text{g L}^{-1} \text{NO}_3^-$ ), and  $\text{CO}_2$  concentrations were moderately undersaturated with respect to the atmosphere. The phytoplankton assemblages were dominated by photosynthetic nanoflagellates. In contrast, the continental shelf and shelf break areas were typically characterized by low nutrient concentrations, high Chl *a* values, and  $\text{CO}_2$

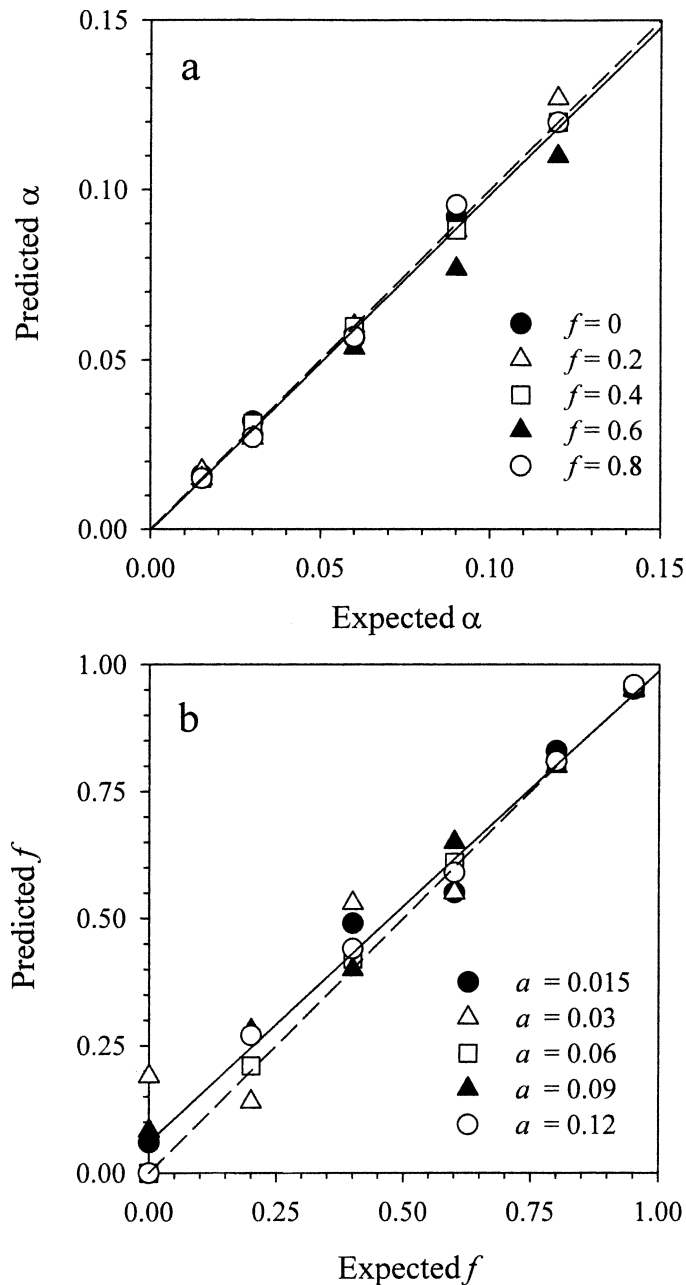


Fig. 2. Test of model-fitting procedure to estimate (a) carbonic anhydrase activity and (b)  $\text{HCO}_3^-$  transport.

concentrations well below atmospheric equilibrium. Large diatoms dominated the phytoplankton populations in these waters, with prevalent genera including *Chaetoceros*, *Pseudonitzschia*, *Fragilariopsis*, *Cylindrotheca*, *Rhizosolenia*, and *Thalassiosira*. Station 10, situated close to the Aleutian islands, exhibited high  $\text{NO}_3^-$  (and presumably  $\text{CO}_2$ ) levels, most likely reflecting the influence of N. Pacific deep water entrainment into the surface. (Detailed information on  $\text{CO}_2$  distributions across the sampling region can be found in Tortell 2005b.) The highest productivity was recorded in the coastal areas and shelf break domain ( $>5 \text{ mg C m}^{-3} \text{ h}^{-1}$ ), while the lowest productivity ( $0.3 \text{ mg C m}^{-3} \text{ h}^{-1}$ ) was observed in the western gyre. In general, calcification

Table 1. Biological and chemical properties of sampling stations.

Station	Nitrate ( $\mu\text{mol L}^{-1}$ )	Phosphate ( $\mu\text{mol L}^{-1}$ )	Silicate ( $\mu\text{mol L}^{-1}$ )	Chl <i>a</i> ( $\mu\text{g L}^{-1}$ )	pCO <sub>2</sub> (Pa)	Primary productivity (mg C m <sup>-3</sup> h <sup>-1</sup> )	Dominant phytoplankton
1	15.1	1.08	13.9	0.68	34.2	0.48±0.05	Nanoflagellates
2	14.7	1.10	16.70	0.73	33.4	0.31±0.03	Nanoflagellates
3	1.33	0.32	—	3.74	27.3	4.4±0.69	Diatoms
4	0.022	0.04	0.81	4	13.6	5.2±0.54	Diatoms
5 (2 $\mu\text{m}$ )	0.10	0.20	1.32	2.39	29.7	3.7±0.19	Nanoflagellates
5 (20 $\mu\text{m}$ )							Diatoms
6 (2 $\mu\text{m}$ )	0.12	0.05	3.58	1.4	33.9	1.18±0.02	Nanoflagellates
6 (20 $\mu\text{m}$ )							Diatoms
7	3.36	0.20	10.1	3.70	22.0	—	Diatoms
8	2.41	0.089	8.6	1.97	23.9	—	Nanoflagellates
9	0.04	0.050	0.85	6.01	—	—	Diatoms
10	15.7	1.23	39.1	5.74	—	—	Nanoflagellates
11	—	—	—	—	—	—	Diatoms

rates were less than 1% of primary productivity at all stations, and there was no apparent relationship between these parameters (calcification data not shown). The chemical and biological properties observed at the sampling sites were comparable to previous studies carried out in the Bering Sea (*see* Loughlin and Ohtani 1999).

*Isotope disequilibrium experiments: in situ assemblages*—Typical isotope disequilibrium results are shown in Fig. 3 for control (i.e., without AZ) experiments at four stations. Model fits to the time-course data showed that direct  $\text{HCO}_3^-$  transport accounted for the majority of C uptake by the phytoplankton at all stations. The fraction of C uptake attributable to direct  $\text{HCO}_3^-$ ,  $f_{\text{HCO}_3^-}$  (hereafter referred to as  $f$ ), ranged from 58% to 86%, with relative errors of  $\pm 1$ –6% (Table 2). Indirect  $\text{HCO}_3^-$  use via eCA expression, modeled as an increase in  $\alpha$ , was also observed. At most stations, modeled  $\alpha$  values (ranging from 0.022 to 0.079) were significantly higher than the uncatalyzed value (0.015), but the apparent enhancement of  $\text{HCO}_3^-$ – $\text{CO}_2$  exchange was relatively modest (Table 2). The highest  $\alpha$  value, 0.079, corresponded to a 5.7-fold enhancement of  $\text{HCO}_3^-$ – $\text{CO}_2$  interconversion rate, while the average for all stations was a 2.4-fold enhancement. At 2 of the 11 stations, modeled  $\alpha$  values were not significantly different from the uncatalyzed rate constant indicating little or no external CA activity. Even after allowing for eCA expression (i.e., increased  $\alpha$ ), the  $^{14}\text{C}$  time-course data could not be adequately fit using a  $\text{CO}_2$ -only uptake model (dashed curves on Fig. 3). This supports the conclusion that direct  $\text{HCO}_3^-$  transport was an essential component of the C acquisition system of all the phytoplankton assemblages.

Size fractionation experiments conducted at two stations (Stas. 5 and 6) revealed differences in C uptake between the  $>2$  and  $>20$   $\mu\text{m}$  components of the phytoplankton assemblages (Table 2). At both stations, the overall species assemblage (i.e.,  $>2$ - $\mu\text{m}$  size fraction) was dominated by nanoflagellates, whereas the  $>20$ - $\mu\text{m}$  size fraction was diatom dominated. External carbonic anhydrase activity ( $\alpha$ ) was higher in the diatom size fraction at both stations; however, the differences were not statistically significant ( $p$

$> 0.05$ ) given the error associated with the parameter estimates. Similarly, estimated values of  $f$  were higher in the  $>20$ - $\mu\text{m}$  fraction at Sta. 5, but the difference was not statistically significant. At Sta. 6,  $f$  values were nearly identical between the two size fractions.

Isotope disequilibrium experiments conducted in the presence of AZ showed that the inhibitor had an observable effect on C uptake by the phytoplankton assemblages. Figure 4 shows a comparison of  $^{14}\text{C}$  time-course data for two stations in the presence and absence of AZ. Despite an increase in the total amount of  $^{14}\text{C}$  assimilated by AZ-treated cells, the inhibitor significantly decreased both the steady-state rate of C uptake ( $V_1$ ) and the fraction of direct  $\text{HCO}_3^-$  transport. Total C uptake rates were reduced by as much as threefold (to 25% of the value observed in control samples), and nearly all of the reduction in C uptake was attributable to a decrease in  $\text{HCO}_3^-$  transport (Fig. 5). Decreased C uptake rates in the presence of the inhibitor were observed even in those assemblages that possessed very low eCA activity (e.g., Sta. 6, Fig. 4b). We interpret these results as evidence of AZ inhibition of the  $\text{HCO}_3^-$  transport system (*see Discussion*), and we thus consider only results from control experiments in the following sections.

*C uptake across environmental gradients*—We examined potential relationships between C uptake by the phytoplankton assemblages and the environmental properties of the sampling stations. No significant correlations were observed between the fraction of direct  $\text{HCO}_3^-$  transport ( $f$ ) and any of the measured chemical or biological properties of each station. High  $f$  values were obtained in both highly productive coastal waters and offshore lower productivity regions, with no apparent relationship to  $\text{CO}_2$  concentrations ( $r = -0.31$ ,  $p > 0.1$ ). Similarly, no significant associations were observed between eCA activity and nutrient concentrations, Chl *a*, or primary productivity. There was, however, an apparent trend toward high activity at low  $\text{CO}_2$  stations (Fig. 6). This relationship was statistically significant for the pooled data set ( $r = -0.85$ ,  $p < 0.01$ ) but was largely influenced by a single station with the highest eCA activity and lowest  $\text{CO}_2$ .

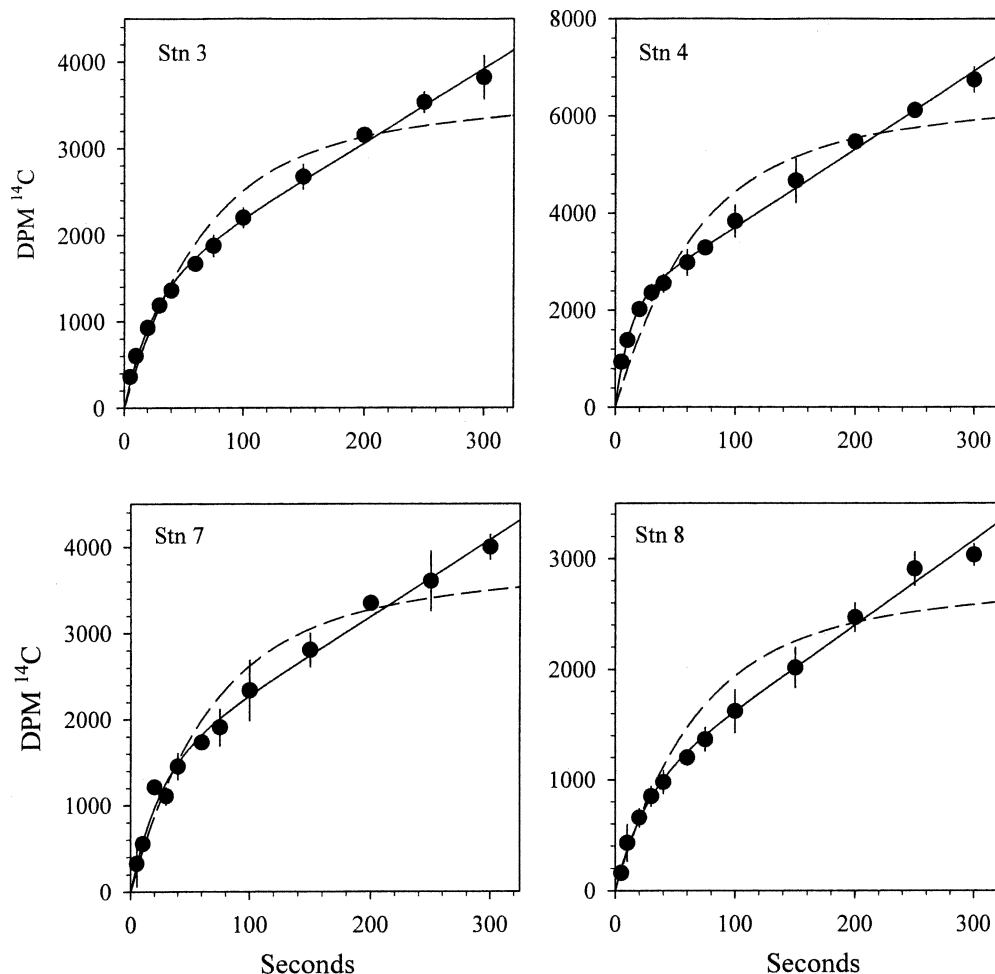


Fig. 3. Time course of  $^{14}\text{C}$  incorporation during isotope disequilibrium experiments for Stas. 3, 4, 7, and 8 in the absence of AZ. The best fit curves were obtained by applying Eq. 1 to the data. The dashed curves represent a  $\text{CO}_2$ -only uptake model fit (i.e.,  $f = 0$ ).

Table 2. Carbon uptake parameters derived from isotope disequilibrium experiments for control (i.e., minus AZ) cells.  $f_{\text{HCO}_3^-}$  is the fraction of total C uptake attributable to direct  $\text{HCO}_3^-$  transport, while  $\alpha$  is the modeled rate constant of  $\text{HCO}_3^-/\text{CO}_2$  exchange, which represents eCA activity. A value of 0.015 is expected for the uncatalyzed rate constant.

Station	$f_{\text{HCO}_3^-}$	$f$ 95% CI*	$\alpha$
1	$0.79 \pm 0.03$	0.73–0.85	$0.029 \pm 0.006$
2	$0.75 \pm 0.02$	0.71–0.79	$0.027 \pm 0.003$
3	$0.83 \pm 0.01$	0.81–0.85	$0.040 \pm 0.005$
4	$0.70 \pm 0.03$	0.64–0.76	$0.079 \pm 0.012$
5 (>2 $\mu\text{m}$ )	$0.72 \pm 0.03$	0.66–0.78	$0.032 \pm 0.004$
5 (>20 $\mu\text{m}$ )	$0.81 \pm 0.02$	0.77–0.85	$0.052 \pm 0.010$
6 (>2 $\mu\text{m}$ )	$0.61 \pm 0.06$	0.49–0.73	$0.022 \pm 0.002$
6 (>20 $\mu\text{m}$ )	$0.58 \pm 0.07$	0.44–0.72	$0.031 \pm 0.005$
7	$0.83 \pm 0.02$	0.79–0.87	$0.041 \pm 0.010$
8	$0.74 \pm 0.04$	0.66–0.82	$0.026 \pm 0.004$
9	$0.87 \pm 0.02$	0.83–0.91	$0.041 \pm 0.010$
10	$0.79 \pm 0.04$	0.71–0.87	$0.020 \pm 0.003^\dagger$
11	$0.86 \pm 0.04$	0.78–0.94	$0.039 \pm 0.019^\dagger$

\* 95% confidence interval, based on twice the standard error of the mean.  
 † Value not significantly different from uncatalyzed rate ( $t < 1.725$ ,  $p > 0.05$ ,  $df = 21$ ).

Primary productivity was highest at this station, and the phytoplankton assemblage was dominated by the diatom *Rhizosolenia* spp. Removal of this station from the analysis eliminated the statistical significance of the eCA– $\text{CO}_2$  correlation ( $r = -0.59$ ,  $p > 0.05$ ). In addition to a potential  $\text{CO}_2$  effect, the taxonomic composition of the phytoplankton assemblages also appeared to influence apparent eCA expression levels. Stations that were dominated by diatoms (3, 4, 7, 9, 11) had significantly higher eCA levels than the flagellate-dominated stations (1, 2, 5, 6, 8, 10) ( $t$ -test,  $p = 0.02$ , triangles in Fig. 6). The diatom-dominated samples mostly occurred over the continental shelf, and the shelf break region in the lowest  $\text{CO}_2$  waters (Stas. 3, 7, 9, and 11), and this imparted a distinct spatial pattern on the distribution of eCA activity across the sampling area.

*CO<sub>2</sub> incubation experiments*—Incubation experiments were conducted to examine the  $\text{CO}_2$ -dependent regulation of the C uptake system. Phytoplankton biomass, nutrient concentrations, growth rates, calcification rates, and primary productivity did not differ significantly between the low (15.2 Pa) and high (76 Pa)  $\text{CO}_2$  incubations.

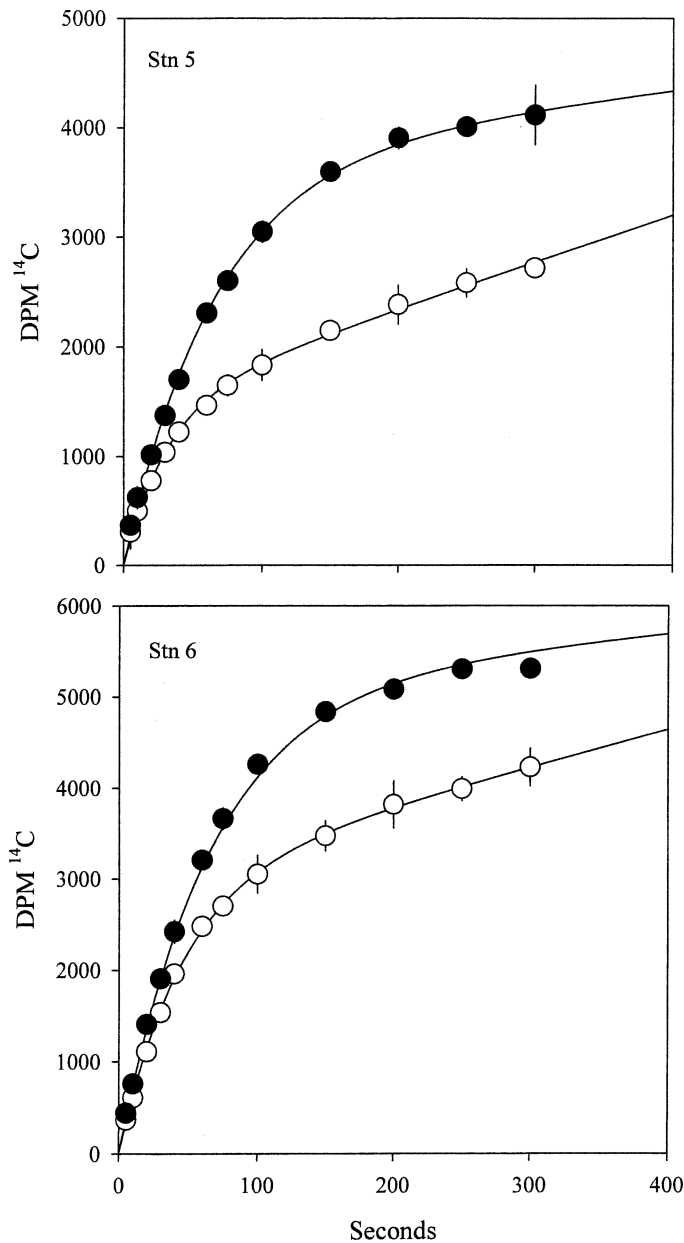


Fig. 4. The effects of Acetazolamide treatment on the time course of  $^{14}\text{C}$  assimilation by phytoplankton assemblages at Stas. 5 and 6. Filled symbols represent AZ-treated samples.

Growth rates were  $0.81 \pm 0.14 \text{ d}^{-1}$  and  $0.88 \pm 0.10 \text{ d}^{-1}$  for the low and high  $\text{CO}_2$  incubations, respectively. Calcification rates were  $<1\%$  of primary productivity in all treatments. A qualitative examination of the phytoplankton assemblages showed no obvious differences in species composition between the low and high  $\text{CO}_2$  incubations at the time when samples were collected for experiments ( $\sim 3 \text{ d}$ ). Both experiments had a mixture of nanoflagellates, dinoflagellates, and thin pennate diatoms, with *Cylindrotheca* sp. being the most prevalent species. Large centric diatoms (*Thalassiosira* spp.) were also found in both  $\text{CO}_2$  treatments of the second incubation experiment. While no large changes in taxonomic composition were observed

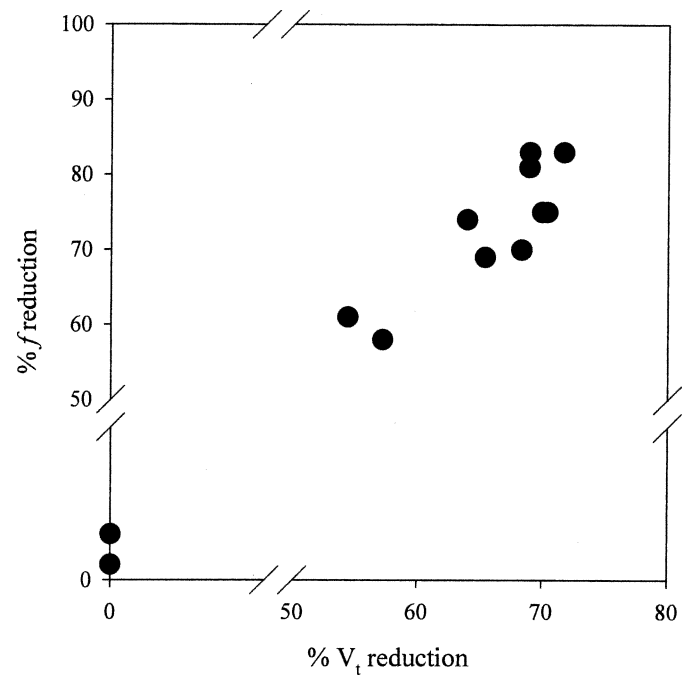


Fig. 5. Relationship between the fractional inhibition of total C uptake ( $V_t$ ) and  $\text{HCO}_3^-$  uptake ( $f_{\text{HCO}_3^-}$ ) caused by AZ additions.

across the  $\text{CO}_2$  treatments, our qualitative analysis does not rule out species-level changes.

Although phytoplankton productivity, growth rate, and biomass did not differ between the low and high  $\text{CO}_2$  treatments, we observed  $\text{CO}_2$ -dependent differences in the relative proportions of  $\text{HCO}_3^-$  and  $\text{CO}_2$  uptake by the phytoplankton assemblages. In both incubations, there was a statistically significant increase in total C uptake rates ( $V_t$ ) in low  $\text{CO}_2$  treatments (Fig. 7; Table 3). The proportion of direct  $\text{HCO}_3^-$  transport also increased at 150 ppm  $\text{CO}_2$ , but this increase was only statistically significant for the first incubation. For both incubation experiments, eCA expression (modeled  $\alpha$  values) decreased under low  $\text{CO}_2$  conditions, but the difference was not statistically different given the parameter errors.

## Discussion

Isotope disequilibrium experiments have proven valuable for the study of C uptake by natural marine phytoplankton assemblages. In our experience, the method is much less laborious than silicone oil centrifugation experiments (e.g., Tortell et al. 2000), and recent work has found excellent agreement between isotope disequilibrium results and mass spectrometric  $\text{CO}_2$  flux analysis (Rost pers. comm.). One advantage of the method, as we have applied it, is its ability to quantify eCA activity in the cell boundary layer without using inhibitors. The principal limitation of the  $^{14}\text{C}$  experiments is an inherent sensitivity bias toward  $\text{CO}_2$  uptake over  $\text{HCO}_3^-$  uptake due to the large relative change in  $\text{CO}_2$  specific activity. Our analysis suggests that low rates of  $\text{HCO}_3^-$  transport ( $\sim <25\%$  of DIC flux) cannot be accurately resolved with this method

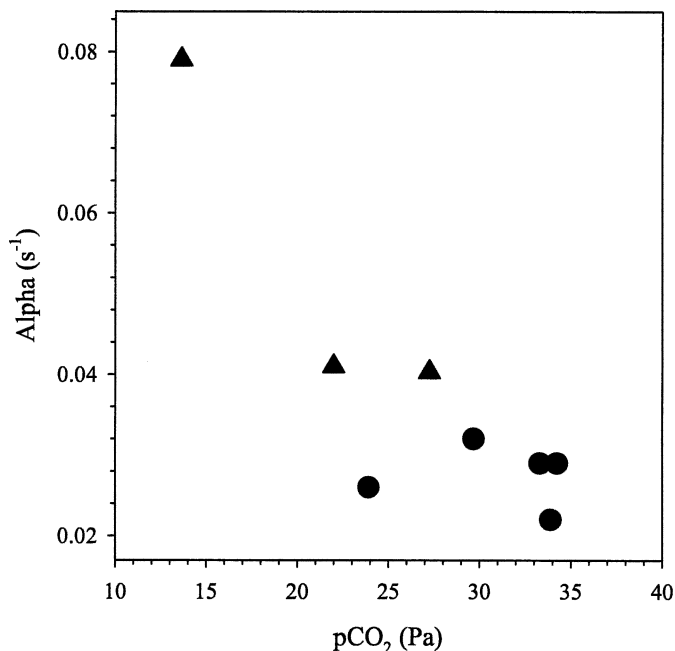


Fig. 6. Relationship between apparent eCA activity and  $p\text{CO}_2$  across the sampling stations. Triangles indicate samples that are diatom dominated, while circles represent nanoflagellate-dominated samples.

(Fig. 2). It should also be noted that the isotope disequilibrium technique provides no information on several important C acquisition parameters (e.g., intracellular DIC concentrations, relative rates of gross C uptake and efflux, and DIC affinity), which are needed to fully characterize the CCM in natural phytoplankton populations (Rost et al. 2003).

*Direct versus indirect  $\text{HCO}_3^-$  use*—Overall, our analysis provides compelling evidence for  $\text{HCO}_3^-$  use through both a direct transport mechanism and via eCA-catalyzed dehydration. Our results, in conjunction with those of previous field studies (Tortell and Morel 2002; Cassar et al. 2004; Tortell et al. 2006), suggest that  $\text{HCO}_3^-$  use is widespread among phytoplankton communities in a variety of oceanic regions. In Bering Sea phytoplankton assemblages, the majority (~60–90%) of  $\text{HCO}_3^-$  use occurred through a direct transport system. This range is consistent with our recent observations in the Subarctic Pacific (Tortell et al. 2006) but somewhat higher than that reported in previous field studies (Tortell and Morel 2002; Cassar et al. 2004). These previous studies have used AZ-treated samples to estimate direct  $\text{HCO}_3^-$  transport. Our results suggest that this inhibitor can significantly affect the  $\text{HCO}_3^-$  transport system, leading to an underestimation of the  $\text{HCO}_3^-$  contribution to total C uptake (Fig. 5). A similar conclusion has been reached based on laboratory studies with the green alga *Chlorella* (Pollock and Colman 2001). Although the molecular mechanism of the AZ effect on  $\text{HCO}_3^-$  transport is not known, it has been interpreted as evidence of a CA-like moiety in the  $\text{HCO}_3^-$  transporter (Pollock and Colman 2001; see also Kaplan and Reinhold 1999). Regardless of the underlying mechanism, we suggest

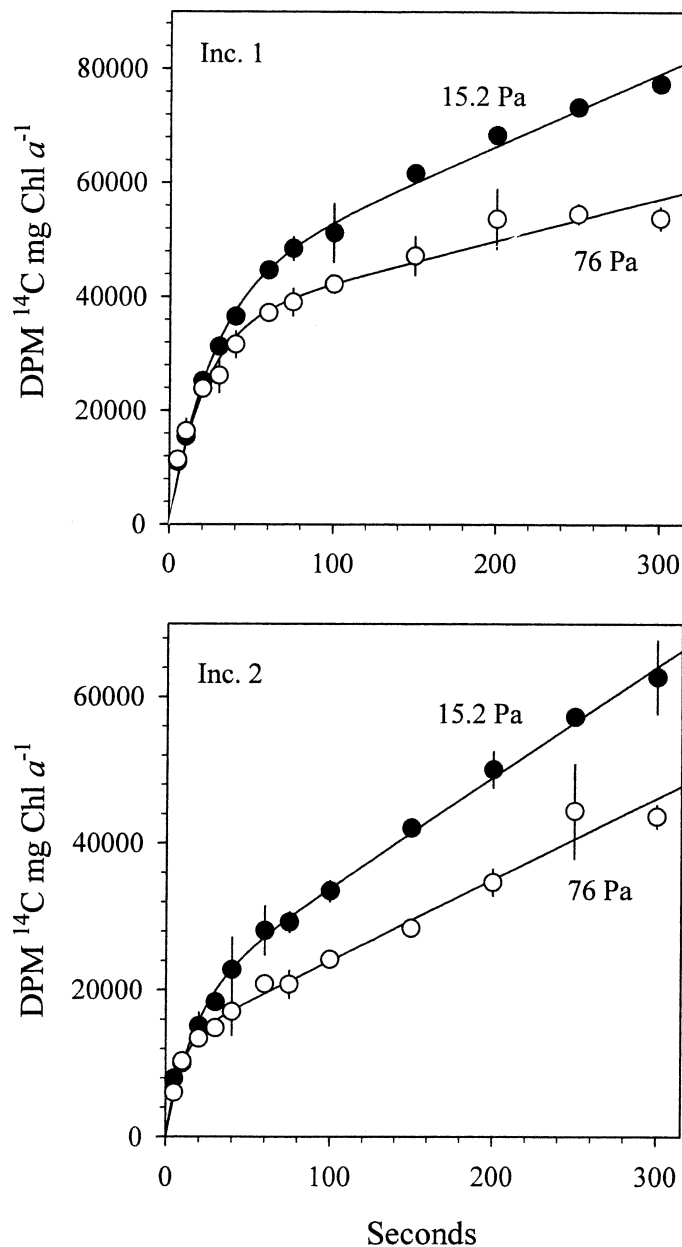


Fig. 7. Effects of  $\text{CO}_2$  manipulations on C uptake in two incubation experiments.

that the results of C uptake experiments with AZ-treated cells should be interpreted with caution.

Laboratory studies indicate that the capacity for  $\text{HCO}_3^-$  transport varies significantly among marine phytoplankton species (Colman et al. 2002). It is thus expected that  $\text{HCO}_3^-$  transport capacity may differ significantly among phytoplankton assemblages with varying taxonomic compositions. Indeed, Tortell and Morel (2002) observed that the proportion of direct  $\text{HCO}_3^-$  transport was highest in cyanobacterial-dominated populations in the equatorial Pacific, lowest in diatom-dominated assemblages, and intermediate in samples containing mixtures of diatoms and nanoflagellates. In contrast, we did not observe any clear differences in  $\text{HCO}_3^-$  transport between diatom- and

Table 3. Carbon uptake parameters determined for phytoplankton in  $\text{CO}_2$  manipulation experiments.

Incubation	$\text{CO}_2$ level (Pa)	$V_t$	$f_{\text{HCO}_3^-}$	$\alpha$
1	15.2	$11.0 \pm 0.70^*$	$0.66 \pm 0.021^\dagger$	$0.042 \pm 0.004$
	76.0	$6.0 \pm 0.78$	$0.42 \pm 0.067$	$0.053 \pm 0.008$
2	15.2	$10.9 \pm 0.57^*$	$0.83 \pm 0.019$	$0.057 \pm 0.011$
	76.0	$7.50 \pm 0.48$	$0.73 \pm 0.060$	$0.097 \pm 0.033$

\*  $p < 0.001$ .†  $p < 0.01$ .

nanoflagellate-dominated phytoplankton assemblages in the Bering Sea. Similarly, Cassar et al. (2004) reported no effects of taxonomic composition (diatom vs. flagellate dominated) on  $\text{HCO}_3^-$  transport by the phytoplankton assemblages in the Southern Ocean. One potential explanation for this apparent lack of taxonomic effects is the covariance between phytoplankton species composition and environmental conditions (e.g.,  $\text{CO}_2$  concentrations). In our Bering Sea survey, stations with the highest diatom abundances were also those with the lowest ambient  $\text{CO}_2$  concentrations and highest primary productivity. These latter two factors could have influenced  $\text{HCO}_3^-$  transport in a manner that obfuscated potential taxonomic effects (*see following*).

In addition to direct  $\text{HCO}_3^-$  transport, we also found evidence for indirect  $\text{HCO}_3^-$  use, based on the eCA-catalyzed enhancement of  $\text{HCO}_3^-$ - $\text{CO}_2$  exchange. However, external carbonic anhydrase appeared to play a relatively minor role in C uptake by the phytoplankton assemblages, catalyzing the rate of  $\text{HCO}_3^-$  dehydration by a factor of  $\sim 5$  at most (Table 2). Similar results have been recently obtained for phytoplankton in the Southern Ocean (Cassar et al. 2004) and Subarctic Pacific (Tortell et al. 2006). In contrast, a previous field study reported significant eCA levels in equatorial Pacific diatom assemblages. Low eCA expression does not appear to be a general characteristic of all marine phytoplankton. Indeed, a number of marine species have been shown to possess high eCA activity when grown in laboratory cultures (Colman and Rotatore 1995; Morel et al. 2002; Rost et al. 2003). To our knowledge, none of the dominant diatom species in our Bering Sea assemblages have been studied in laboratory cultures, and we are thus unable to determine whether eCA activity is a characteristic of these species or a result of some environmental factor(s). In particular, low dissolved Zn concentrations in the offshore waters of the Bering Sea (Bruland pers. comm.) and the higher solubility of  $\text{CO}_2$  in cold high latitude regions might act to diminish eCA expression (*see* Morel et al. 2002). At present, it would appear that low eCA expression may be characteristic of many marine phytoplankton assemblages in situ, though more data are needed to critically examine this.

Despite relatively low expression levels, we were able to discern significant differences in eCA activity among Bering Sea phytoplankton assemblages of varying taxonomic compositions, with higher eCA activity in diatom-dominated assemblages relative to flagellate-dominated assemblages. The interpretation of this result is complicated, however, by a  $\text{CO}_2$  gradient that was superimposed

upon the taxonomic differences among stations; diatom-dominated assemblages were found in low  $\text{CO}_2$  waters. Size fractionation experiments help to separate taxonomic and environmental influences by examining the various components of a single phytoplankton assemblage. At the two stations where size fractionation was performed, the modeled eCA activity was higher for diatom-dominated samples ( $>20 \mu\text{m}$ ), yet this difference was not statistically significant. Despite the lack of a statistically significant effect, this result is consistent with higher eCA levels in diatoms. Previous studies have also reported elevated eCA activity in diatom-dominated assemblages in the equatorial Pacific (Tortell and Morel 2002) and in coastal waters of British Columbia, Canada (Hobson et al. 2001). In contrast, Cassar et al. observed no apparent differences in eCA activity among Southern Ocean phytoplankton communities dominated by either diatoms or nanoflagellates. Higher eCA activity observed in (at least some) diatom-dominated communities may reflect the lower surface area to volume ratio of large cells, which leads to a greater depletion of  $\text{CO}_2$  in the diffusive boundary layer (Riebesell and Wolf-Gladrow 2002).

*CO<sub>2</sub> regulation of the CCM*—Given the resource costs associated with the CCM (e.g., energy, proteins, and trace metals; Raven and Johnston 1991), it is expected that DIC acquisition by phytoplankton should be regulated by cellular C requirements and ambient C supply. Many laboratory studies have reported a reduction in  $\text{HCO}_3^-$  transport under high  $\text{CO}_2$  conditions (*see* Colman et al. 2002), yet much of this work has been conducted using extremely high  $\text{CO}_2$  treatments (e.g.,  $\sim 5,000$  Pa  $\text{CO}_2$ ) the environmental relevance of which is questionable. Recent work with several marine algae has confirmed that  $\text{HCO}_3^-$  transport is tightly regulated by  $\text{CO}_2$  concentrations from 18.2 to 182 Pa (Rost et al. 2003). In addition, several field studies have provided evidence for the  $\text{CO}_2$ -dependent regulation of C uptake by natural marine phytoplankton assemblages (Tortell et al. 2000; Tortell and Morel 2002; Cassar et al. 2004).

In our sampling of in situ Bering Sea phytoplankton assemblages, we did not observe any evidence for a  $\text{CO}_2$ -dependent regulation of  $\text{HCO}_3^-$  transport. We found high rates of  $\text{HCO}_3^-$  transport across all  $\text{CO}_2$  concentrations and productivity levels. Similarly, Cassar et al. (2004) reported that Fe fertilization did not increase direct  $\text{HCO}_3^-$  transport by Southern Ocean phytoplankton despite a significant increase in primary productivity and a decrease in ambient  $\text{CO}_2$  concentrations. These observations suggest that the  $\text{HCO}_3^-$  transport system is constitutively expressed by natural marine phytoplankton assemblages as has been previously observed in laboratory cultures of many phytoplankton species including marine diatoms (e.g., Matsuda et al. 2001).

Unlike  $\text{HCO}_3^-$  transport, eCA activity showed some relationship with  $\text{CO}_2$  concentrations in the in situ sampling transects, with the highest activity observed in low  $\text{CO}_2$  waters. As discussed above, however, the interpretation of results obtained across sampling stations is complicated by the simultaneous change of species composition and environmental conditions. More specifically,

low CO<sub>2</sub> stations where phytoplankton showed the highest eCA activities were also those where diatoms were predominant (Fig. 6). It is therefore unclear whether the gradient in eCA activity reflects the change in CO<sub>2</sub> levels or the taxonomic composition of the phytoplankton assemblages. In our recent sampling of Subarctic Pacific phytoplankton assemblages (Tortell et al. 2006), we have found independent evidence for a CO<sub>2</sub> effect on CA expression. Total cellular CA (both intracellular, iCA, and external, eCA) was very tightly correlated to ambient CO<sub>2</sub> concentrations, with high expression observed in low CO<sub>2</sub> waters. The measurements did not allow us to distinguish the relative contributions of iCA and eCA to the total activity, but the phytoplankton appeared to possess low eCA levels. The results thus point to a CO<sub>2</sub> regulation of intracellular CA activity. As with the Bering Sea results, however, this conclusion is subject to uncertainty given the probable covariance of CO<sub>2</sub> concentrations and phytoplankton species composition. Thus, in situ field sampling alone cannot provide unambiguous information on the regulation of C uptake by environmental variables.

Incubation experiments provide a more direct means of examining the effects of CO<sub>2</sub> on phytoplankton assemblages. In our relatively short experiments, phytoplankton species composition did not change significantly, yet we found strong evidence for CO<sub>2</sub> regulation of the C uptake system. In two independent experiments, low CO<sub>2</sub> conditions increased the total C uptake rates ( $V_T$ ) of phytoplankton and the relative contribution of direct HCO<sub>3</sub><sup>-</sup> transport ( $f$ ) (Fig. 7; Table 3). The upregulation of C transport rates at low CO<sub>2</sub> has been observed in previous CO<sub>2</sub> manipulation experiments (Tortell et al. 2000). This effect may be associated with a rise in the cellular affinities for CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> (Rost et al. 2003), but our data do not allow us to assess this directly. It is important to note that despite a significant reduction, HCO<sub>3</sub><sup>-</sup> transport still accounted for over 50% of the C uptake by high CO<sub>2</sub> assemblages. This suggests that the HCO<sub>3</sub><sup>-</sup> transport system should never be fully repressed under any oceanic conditions.

While HCO<sub>3</sub><sup>-</sup> transport seemed to be upregulated in low CO<sub>2</sub> incubation bottles, eCA activity appeared to decrease with decreasing CO<sub>2</sub>, although the observed changes were not statistically significant. This result seems at odds with our observations (Fig. 6) and those of others indicating high eCA activity under low CO<sub>2</sub> conditions. Typically, eCA activity has been reported to increase in response to low CO<sub>2</sub> levels both in laboratory and field experiments (e.g., Elzenga et al. 2000; Lane and Morel 2000; Tortell and Morel 2002). While the enhancement of eCA activity under low CO<sub>2</sub> appears to be a robust observation, the physiological role of this enzyme under low CO<sub>2</sub> conditions is not entirely clear. Theoretical considerations suggest that the enzyme provides only a small enhancement of cellular C fluxes at low ambient CO<sub>2</sub> concentrations (Riebesell and Wolf-Gladrow 2002).

*Oceanographic implications*—The widespread occurrence of HCO<sub>3</sub><sup>-</sup> transport suggests that CO<sub>2</sub> concentrations may not limit the growth rates of marine phytoplankton assemblages, as initially suggested by Riebesell et al.

(1993). Indeed, the results of a number of incubation experiments (Tortell et al. 1997, 2000, 2002, this study) demonstrate that bulk phytoplankton growth rates are relatively insensitive to CO<sub>2</sub> concentrations ranging from 10.1 to 81 Pa (approximately 3–30 μmol L<sup>-1</sup> aqueous CO<sub>2</sub>). Over longer timescales, however, CO<sub>2</sub> concentrations may affect the taxonomic composition of phytoplankton communities (Tortell et al. 2002). Such taxonomic shifts are likely influenced by the physiological mechanisms of inorganic C use by individual species, and it is thus necessary to fully characterize the CCM of natural phytoplankton assemblages to predict how they will respond to future changes in CO<sub>2</sub> levels. In addition to quantifying the relative proportions of HCO<sub>3</sub><sup>-</sup> and CO<sub>2</sub> used by cells, future field studies should examine the kinetics (i.e., maximum transport rates and substrate affinities) of the C transport system (e.g., Rost et al. 2003).

The transport of HCO<sub>3</sub><sup>-</sup> by oceanic phytoplankton also affects the C isotope signature of marine organic matter by altering the relationship between CO<sub>2</sub> concentrations and cellular isotope fractionation (Fielding et al. 1997; Keller and Morel 1999). This occurs, in part, because the C isotope composition of HCO<sub>3</sub><sup>-</sup> is approximately 10‰ heavier (i.e., enriched in <sup>13</sup>C) than CO<sub>2</sub> (Mook et al. 1974). As a result, direct HCO<sub>3</sub><sup>-</sup> transport may lower the apparent isotope fractionation factor of phytoplankton by providing an isotopically heavy C substrate for intracellular assimilation. Indeed, previous authors have invoked direct HCO<sub>3</sub><sup>-</sup> transport to help explain the low <sup>13</sup>C values often observed in highly productive diatom-dominated waters (Fry and Wainwright 1991). In contrast, indirect HCO<sub>3</sub><sup>-</sup> use (via eCA-catalyzed HCO<sub>3</sub><sup>-</sup>–CO<sub>2</sub> exchange) does not provide the same heavy C, since the CO<sub>2</sub> produced via CA-catalyzed dehydration has the same isotopic composition as the bulk CO<sub>2</sub> pool (i.e., ~10‰ lighter than HCO<sub>3</sub><sup>-</sup>; see Goericke et al. 1994). Even though the exact physiological details remain poorly understood, all of the available field data provide compelling evidence that the simple CO<sub>2</sub> diffusion model cannot be used to explain C isotope fractionation by marine phytoplankton assemblages. While some individual species may conform well to this theoretical model (e.g., Laws et al. 1995), they may be poor representatives of most phytoplankton species in situ. This highlights the importance of conducting field sampling and experimentation with natural phytoplankton assemblages.

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Received: 7 July 2005

Accepted: 18 December 2005

Amended: 13 April 2006