

Inorganic carbon uptake and intracellular assimilation by subarctic Pacific phytoplankton assemblages

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Abstract

We report the results of inorganic carbon (C) uptake experiments and activity measurements for carbonic anhydrase, ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), and phosphoenolpyruvate carboxylase (PEPC) in offshore and coastal regions of the eastern subarctic Pacific Ocean. HCO_3^- was the dominant source of inorganic C taken up by phytoplankton at all sampling locations, accounting for ~60–90% of total C uptake. The uptake of HCO_3^- occurred primarily through a direct transport system, while indirect HCO_3^- use, mediated by extracellular carbonic anhydrase (eCA), played a minor role in the C uptake system. Direct HCO_3^- transport and eCA activity were not related to ambient nutrient or CO_2 concentrations or to phytoplankton biomass (chlorophyll *a* [Chl *a*]) or primary productivity. There was significant variability in the biomass-normalized activities of Rubisco, PEPC, and total (intracellular and extracellular) carbonic anhydrase. The activities of all of the enzymes measured exhibited significant correlations with both CO_2 concentrations and Chl *a*. PEPC activity averaged 20% of Rubisco activity (range 0.5–110%), and the PEPC:Rubisco ratio was positively correlated with CO_2 concentrations and negatively correlated with Chl *a*. Carbonic anhydrase activity was strongly anticorrelated with CO_2 and positively correlated with Chl *a*. The results provide evidence for the importance of CO_2 -regulated carbon concentrating mechanisms in marine waters.

Laboratory studies have documented the existence of inorganic carbon concentrating mechanisms (CCMs) in a variety of marine algal and cyanobacterial species (*see* Kaplan and Reinhold 1999, and Colman et al. 2002 for recent reviews). Although the molecular pathways have not been fully resolved, it is clear that CCMs enable cells to saturate carbon (C) fixation by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) despite the poor catalytic efficiency of this enzyme (Spreitzer and Salvucci 2002) and the relatively low CO_2 concentrations in seawater. The CCM consists of a high affinity uptake system for inorganic C and the intracellular accumulation of CO_2 in the vicinity of Rubisco. Many phytoplankton species appear to use the abundant HCO_3^- ion as a C source, through either a direct transport system or an indirect uptake pathway in which carbonic anhydrase (CA) catalyzes the otherwise slow dehydration of HCO_3^- to CO_2 in the cell boundary layer (Sültemeyer et al. 1993; Badger 2004). Recent evidence also suggests that some phytoplankton species may possess a C_4 photosynthetic pathway in which intracellular HCO_3^- is converted to an

organic acid via phosphoenolpyruvate carboxylase (PEPC), and CO_2 is subsequently liberated by decarboxylation in close proximity to Rubisco (Reinfeldt et al. 2000). By regulating the activity of the C transport system, Rubisco, carbonic anhydrase, and potentially PEPC, phytoplankton species may optimize growth over a wide range of ambient CO_2 concentrations.

While laboratory experiments have provided detailed information on the mechanisms of carbon acquisition by individual phytoplankton species, relatively little is known about C acquisition by marine phytoplankton populations in the oceans. Indeed, no information on C uptake is currently available for the vast majority of phytoplankton species found in marine waters. For those species that have been studied in laboratory cultures, results may be difficult to extrapolate to the field since phytoplankton often experience in situ limitation by a variety of factors (e.g., light, macronutrients, and trace metals) that may constrain C acquisition (Morel et al. 1994; Beardall et al. 1998).

Several recent field studies have examined C acquisition by phytoplankton assemblages in seawater. The earliest of these demonstrated the presence of CO_2 -regulated carbon concentrating mechanisms (Tortell et al. 1997, 2000), while more recent work has provided quantitative estimates of the contributions of HCO_3^- and CO_2 to cellular C uptake (Tortell and Morel 2002; Cassar et al. 2004). These studies have provided an important first step, but results from a much broader range of oceanic regions are needed before any generalizations can be made about the mechanisms of C acquisition by phytoplankton in the sea. In a companion

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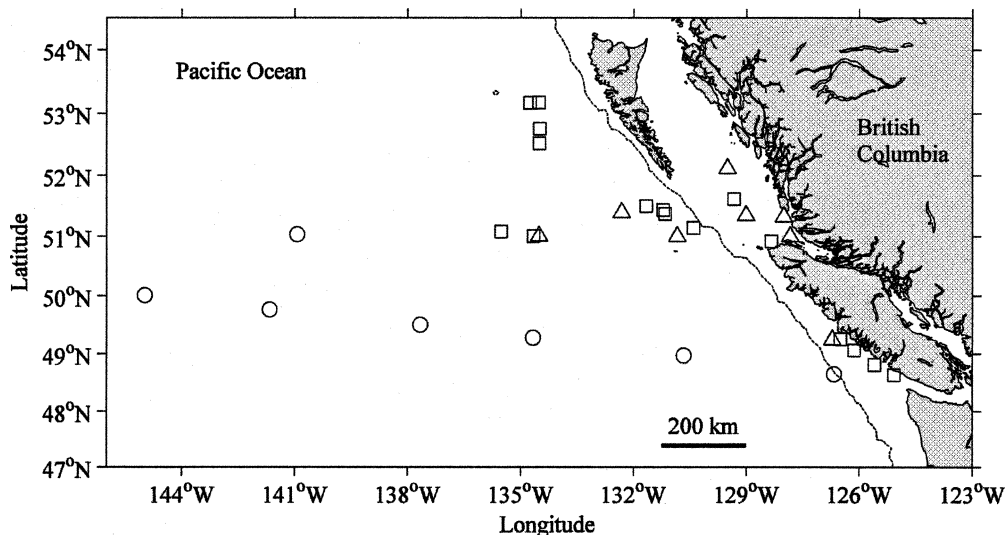


Fig. 1. Location of sampling stations off the west coast of British Columbia, Canada. Different symbols indicate the experiments and sampling that was conducted at each station. Circles, isotope disequilibrium only; squares, enzyme measurements only; triangles, both isotope disequilibrium experiments and enzyme measurements. The dotted line running parallel to the coast represents the 1,000-m isobath.

paper (Martin and Tortell 2006), we present results from isotope disequilibrium experiments showing that HCO_3^- is the main inorganic C source for phytoplankton populations in the southeast Bering Sea and that C uptake by phytoplankton can be regulated by CO_2 concentrations. In this article, we present data from C uptake studies conducted in coastal and offshore waters of the subarctic Pacific. Our results demonstrate that direct HCO_3^- transport dominates C uptake by phytoplankton across all sampling regions despite large differences in total primary productivity, algal biomass, and CO_2 concentrations. We also present activity measurements of key enzymes in the C acquisition system—Rubisco, carbonic anhydrase, and PEPC—which provide insight into the biochemical mechanisms of intracellular C assimilation and its regulation by ambient CO_2 concentrations.

Materials and methods

Sampling and ancillary measurements—Sampling and experiments were conducted on board the Canadian Coast Guard vessel *John P. Tully* in June and August of 2004. Seawater samples were collected at a variety of locations (Fig. 1) including both productive coastal waters and Fe-limited high-nutrient low-chlorophyll (HNLC) regions of the central Gulf of Alaska (Boyd and Harrison 1999). In coastal waters, surface hydrography is highly variable due to small-scale physical fronts, river input, and local upwelling (Crawford and Thomson 1991), and this physical variability is associated with strong gradients in biological productivity and CO_2 concentrations (see Tortell 2005). In contrast, offshore HNLC regions show much less seasonal variability in phytoplankton standing stocks and productivity (Boyd and Harrison 1999). Open ocean phytoplankton assemblages of the subarctic Pacific are typically dominated by

nanoflagellates with some pennate diatoms (Booth 1988), while large centric diatoms are the dominant phytoplankton group during spring and early summer in the high-productivity continental shelf regions (Mackas 1984).

Nutrient concentrations at most stations were determined by shipboard autoanalyzer, while total chlorophyll *a* (Chl *a*) was determined by extraction of GF/F filter samples (0.7- μm nominal pore size) in 90% acetone followed by fluorometric analysis (Parsons et al. 1984). At some stations, pCO_2 was measured by underway membrane inlet mass spectrometry (Tortell 2005). Owing to instrument problems, CO_2 data are not available for all stations. Primary productivity at selected sites was measured as described by Martin and Tortell (2006).

Seawater samples were collected from either the subsurface Chl *a* maximum (typically 10–40 m) using Niskin bottles, or from ~ 3 m depth using the ship's continuous seawater supply. For Niskin bottle samples, phytoplankton were concentrated by in-line gravity filtration onto 47-mm, 2- μm -pore size polycarbonate membranes. In many cases, the entire contents of a Niskin bottle (~ 10 liters) were collected onto a single filter. For surface-water samples, a 25-liter carboy was filled from the seawater line and pressurized with air to ~ 140 kPa to force water through a stirred cell concentrator chamber (Amicon) onto a 47-mm filter. Samples for C uptake experiments were concentrated onto 2- μm polycarbonate membranes, while enzyme samples were collected onto glass-fiber filters (GF/F, nominal pore size 0.7 μm). Enzyme samples were immediately frozen in liquid nitrogen for transport back to the laboratory.

Enzyme activity measurements—Enzyme activities in frozen field samples were determined approximately 3 months after the cruise. For analysis, frozen filters were

placed into 3 mL of an ice-cold extraction/assay buffer and homogenized in a glass grinding tube with a rotating Teflon plunger. The buffer, modified from MacIntyre et al. (1997), contained 50 mmol L⁻¹ bicine (pH 7.8), 1 mmol L⁻¹ ethylenediaminetetraacetic acid (EDTA), 10 mmol L⁻¹ MgCl₂, 1.5 mol L⁻¹ glycerol, 10 mmol L⁻¹ NaHCO₃⁻, 5 mg L⁻¹ bovine serum albumen (BSA), 5 mmol L⁻¹ dithiotriitol (DTT), and 0.1% Triton-X. When no visible pieces of intact filter remained, samples were transferred into 5-mL metal tubes on ice and sonicated with a microtip at 70% duty cycle for several 30-s intervals. Crude cell extracts were then clarified by centrifugation at 14,000 × g for 30 s, and the supernatants retained for enzyme assays. The final concentration of Chl *a* in extracts ranged from ~1 to 10 μg mL⁻¹.

The activities of Rubisco and PEPC were determined using ¹⁴C-based assays modified from previous studies (Descolas-Gros and Oriol 1992; MacIntyre et al. 1997). The assays measure the rate of ¹⁴C incorporation into organic (acid stable) carbon products following the addition of H¹⁴CO₃⁻/¹⁴CO₂ and ribulose biphosphate (RuBP) or phosphoenolpyruvate (PEP), respectively. Briefly, seven 200-μL aliquots were removed from each sample and dispensed into polypropylene microfuge tubes; three replicates each for Rubisco and PEPC activity and one blank. Following a 15-min dark preincubation period to allow for the depletion of any residual RuBP and PEP pools, 20 μL of either RuBP stock (23 mmol L⁻¹), PEP stock (50 mmol L⁻¹), or sterile water (for blanks) was added to the tubes. Substrate stocks were prepared in sterile water and stored at -20°C. After a 3-min incubation at room temperature, 1.33 GBq of NaH¹⁴CO₃⁻ (NEN-DuPont, 0.133 GBq μL⁻¹, 6,660 GBq mmol⁻¹) was added to all tubes to initiate ¹⁴C fixation. After 30 min, reactions were terminated by the addition of 100 μL 6 mol L⁻¹ HCl. (Previous work has shown that the time course of ¹⁴C fixation in vitro by Rubisco and PEPC is linear for over 1 h at substrate concentrations similar to those used in this study; Glover and Morris 1979.) The initiation and termination of all reactions was staggered so that all samples were incubated with substrate and ¹⁴C for the same length of time. Following the addition of acid to samples, tubes were placed in a fume hood on a shaker table and left to degas unfixed ¹⁴C for at least 12 h. Degassed samples were transferred into 7-mL scintillation vials, and 5 mL of scintillation cocktail (Fisher Scintisafe 50%) was added. Radioactivity was measured using a Beckman Coulter LS6500 scintillation counter with correction for quenching and counting blanks. Radioactivity in the blank tubes (¹⁴C added without substrates) was subtracted from all samples.

The activity of total carbonic anhydrase in cell extracts was measured using a membrane inlet mass spectrometer to monitor the rate of ¹⁸O exchange between CO₂ and HCO₃⁻ in a closed system (see Miller et al. 1997). Doubly labeled CO₂ (¹³C¹⁸O¹⁸O) was prepared by dissolving 50 mg of K¹³CO₃²⁻ (99 atom % enrichment; Sigma) into 1 mL of H₂¹⁸O (98 atom % enrichment; Medical Isotopes) and heating in a gas-tight vial at 60°C for 4 h. To initiate experiments, 10 μL of the isotope spike solution was injected into 2 mL of cell lysate or buffer blank in

a thermostated (15°C), stoppered chamber connected to a quadrupole mass spectrometer via a silicone membrane interface. The rate of ¹⁸O exchange was measured by following the concentrations of ¹³C¹⁸O¹⁸O (mass 49), ¹³C¹⁸O¹⁶O (mass 47), and ¹³C¹⁶O¹⁶O (mass 45) for 5 to 10 min and plotting the logarithmic ¹⁸O atom fraction (log [49/(45 + 47 + 49)]) against time. Activity units (*U*) were calculated from the difference in ¹⁸O exchange rates between cell lysate samples (*S*) and blanks (*B*): $U = (S - B)/B$. The CA activity measured in cell extracts represents the sum of intracellular and periplasmic activities. We also estimated periplasmic CA activity from the kinetics of ¹⁴C uptake by cells in vivo (see following).

Enzyme activities were normalized to the biomass of cell lysates as measured by either total protein or Chl *a* content in 100-μL aliquots. Total protein was measured using a commercial assay kit (BioRad), while Chl *a* was measured fluorometrically following extraction in -20°C acetone (Parsons et al. 1984). As expected, we found a significant correlation between the total Chl *a* and protein contents of cell lysates ($r^2 = 0.80$, $p < 0.0001$), with a mean protein: Chl *a* ratio of $31.1 \pm 15.3 \mu\text{g} : \mu\text{g}$. This ratio is similar to that reported by Montagnes et al. (1994). We report our enzyme activities normalized to total protein but note that the observed patterns apply equally to Chl *a*-normalized data.

Inorganic C uptake measurements—Inorganic carbon uptake experiments were conducted using the isotope disequilibrium technique (Espie and Colman 1986). The theory and methodology of this technique are discussed in detail in previous articles (Espie and Colman 1986; Elzenga et al. 2000; Tortell and Morel 2002). We followed the protocol of Martin and Tortell (2006) but conducted the experiments at 15°C (with a concomitant change in sampling times). We used the empirical methods of Martin and Tortell (2006) to estimate the fraction of HCO₃⁻ transport and extracellular CA activity from the kinetic time course data.

Results

Biological and hydrographic setting—Table 1 provides information on the distribution of nutrients and phytoplankton biomass, primary productivity, and CO₂ concentrations across the sampling sites (note that not all measurements are available for each station). Chl *a* concentrations differed by more than an order of magnitude across the sampling area from 0.24 μg L⁻¹ in offshore waters to 5.5 μg L⁻¹ in coastal regions. Primary productivity ranged by two orders of magnitude, from 0.55 to >50 mg C m⁻³ h⁻¹, while NO₃⁻ varied from undetectable (<0.1 μmol L⁻¹) to 17.6 μmol L⁻¹, and CO₂ ranged from 17.6 to 37.7 Pa ([CO₂] aq 6.8–14.5 μmol⁻¹). (Note that 1 Pa = 9.87 ppm = 9.87 μatm.)

Carbon uptake parameters—Typical data obtained from isotope disequilibrium experiments are shown, for three stations, in Fig. 2. Nonlinear regression was used to estimate both the relative contributions of CO₂ and HCO₃⁻ to total inorganic C uptake by the phytoplankton

Table 1. Hydrographic and biological characteristics of the sampling stations.

Station	Samples*	Lat. (°N)	Long. (°E)	Sampling depth (m)	Nitrate ($\mu\text{mol L}^{-1}$)	Chl <i>a</i> ($\mu\text{g L}^{-1}$)	pCO ₂ (Pa)	Primary productivity ($\text{mg C m}^{-3} \text{ h}^{-1}$)
P4	ID	48 65	126 67	15	0.1	0.52	—	—
P12	ID	48 97	130 66	15	2.9	0.55	—	0.97
P16	ID	49 28	134 66	25	7.2	0.38	—	0.74
P19	ID	49 50	137 66	30	—	—	—	0.80
P23	ID	49 77	141 66	30	—	0.28	—	0.77
P26	ID	50 01	144 97	30	14.7	0.32	—	0.89
R17	ID	51 03	140 93	30	8.4	0.24	—	0.55
U6	E	51 08	135 51	3	3.5	0.51	34.2	—
U7	E	51 00	134 65	3	2.8	0.45	32.7	—
U8	E+ID	51 00	134 50	3	1.6	1.13	32.0	—
U9	E	52 52	134 50	3	3.2	0.52	36.3	—
U10	E	52 75	134 49	3	3.1	0.68	34.7	—
U11	E	53 17	134 51	3	5.3	0.26	37.7	—
U12	E	53 17	134 74	3	4.3	0.32	36.8	11.68
U13	E+ID	51 40	132 31	3	0.1	1.79	31.8	52.29
U14	E	51 50	131 66	3	0.1	1.12	28.5	—
U15	E	51 37	131 16	3	3.5	0.53	33.1	—
U16	E	51 14	130 40	3	0.1	0.82	32.1	—
U17	E+ID	51 33	127 99	3	0.1	2.82	17.6	—
U20	E	50 91	128 33	3	0.1	4.43	20.9	—
U21	E	49 26	126 50	3	—	1.28	28.2	—
U22	E	49 06	126 13	3	—	—	—	—
U23	E	48 81	125 59	3	—	—	—	—
U24	E	48 64	125 07	3	—	—	—	—
GB5	E	51 62	129 33	20	5.7	1.96	—	—
MB2	E	51 43	131 21	20	5.5	0.77	—	—
SP4	E+ID	52 12	129 49	10	0.2	1.5	—	—
SS2.5	E+ID	51 00	130 83	20	3.5	0.96	—	—
CPE	E+ID	51 00	127 83	3	17.6	5.5	—	—
SS6	E+ID	51 35	129 00	15	4.4	1.37	—	—
G3	E+ID	49 25	126 71	15	1.1	—	—	—

* ID, isotope disequilibrium experiments; E, enzyme activity measurements.

assemblages and the activity of external CA (see Martin and Tortell 2006 for details of the analysis and interpretation). In the discussion that follows, f refers to the fraction of total C uptake attributable to direct HCO_3^- transport, while α represents the rate constant of $\text{HCO}_3^-/\text{CO}_2$ exchange that reflects eCA activity. The analysis indicated that direct HCO_3^- transport was the dominant mechanism of carbon uptake by phytoplankton at all sampling stations. In no case could the time course data be adequately fit with a CO_2 -only uptake model (i.e., $f = 0$; Fig. 2 dashed line), even after allowing for eCA activity. Estimated HCO_3^- transport, f , ranged from 62% to 91% of total C uptake across the sampling stations, and the standard error of the estimates was typically less than 10% (Table 2).

Indirect HCO_3^- use (i.e., carbonic anhydrase-catalyzed HCO_3^- dehydration coupled to CO_2 uptake) was also observed in some of the sampled phytoplankton assemblages. At 5 of the 15 stations, apparent rate constants for $\text{HCO}_3^-/\text{CO}_2$ equilibration (α) derived from the analysis were significantly (t -test, $p < 0.05$, $df = 21$) greater than the uncatalyzed value, indicating that phytoplankton possessed external carbonic anhydrase activity. However, the cata-

lytic enhancement of HCO_3^- dehydration rates was modest, with a maximum increase in the rate constant of 2.6-fold relative to the uncatalyzed value. At the other 10 stations, modeled α values were not significantly different from the uncatalyzed rate constant, indicating little or no external CA activity. There was no significant relationship between eCA expression (α) and the fraction of direct HCO_3^- transport (f) ($r = -0.170$, $p > 0.1$, $n = 15$).

Carbon uptake parameters did not show any strong environmental patterns across the sampling region. There were no clear differences in direct HCO_3^- transport between coastal and open ocean phytoplankton assemblages. Although the lowest f values were observed in the offshore stations P12, P16, and P19, the parameter estimates for these stations were not statistically different from those of most coastal stations. Moreover, three other offshore stations, P23, P26, and R17, had high f values that were nearly identical to those measured in coastal phytoplankton assemblages. There was a weak positive correlation between phytoplankton biomass (Chl *a*) and f values ($r = 0.52$, $n = 15$), though this correlation was only marginally significant at the 95% level ($p = 0.055$). In contrast, there was no relationship between eCA expression

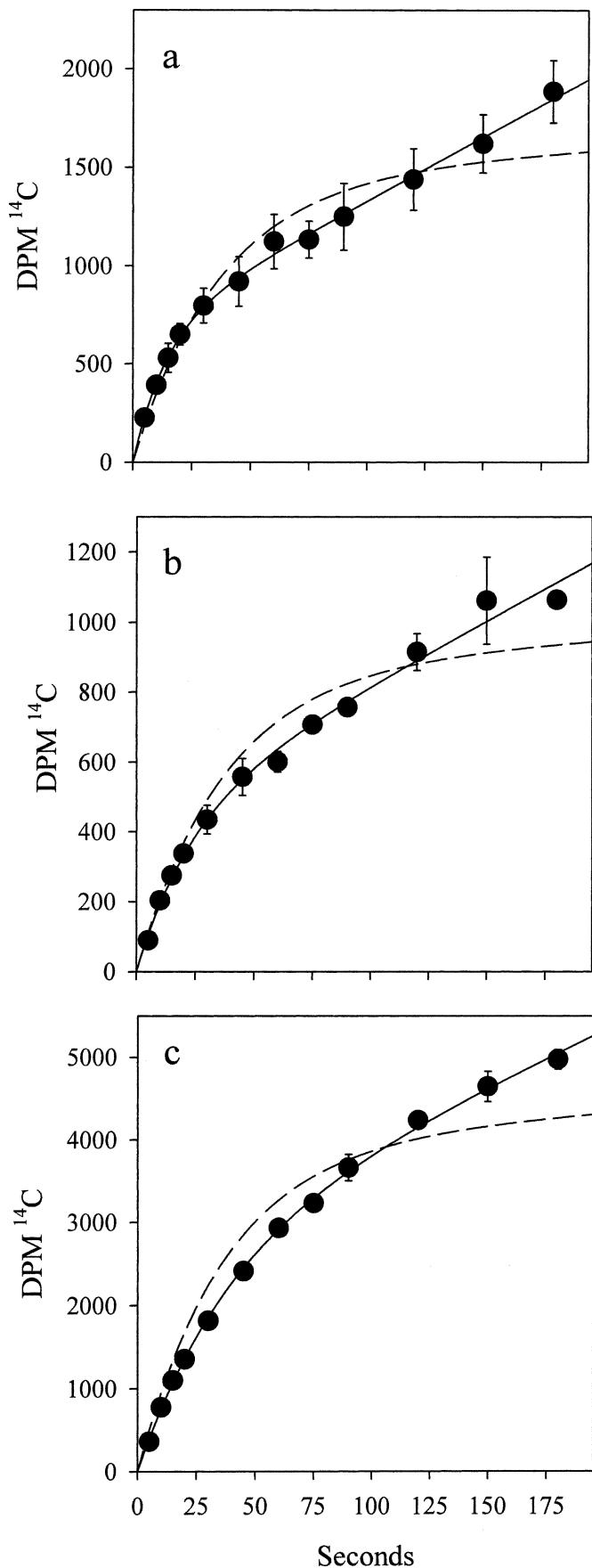


Table 2. Results of isotope disequilibrium modeling. f HCO_3^- is the fraction of total C uptake attributable to direct HCO_3^- transport, and α is the apparent rate constant of $\text{HCO}_3^-/\text{CO}_2$ equilibration. The uncatalyzed rate constant is $0.027 \text{ (s}^{-1}\text{)}$.

Station	$f \text{HCO}_3^-$	f 95% C.I.	$\alpha \text{ (s}^{-1}\text{)}$
P4	0.79 ± 0.02	0.75–0.83	$0.054 \pm 0.010^*$
P12	0.65 ± 0.05	0.55–0.75	$0.046 \pm 0.006^*$
P16	0.65 ± 0.09	0.47–0.83	$0.041 \pm 0.008^*$
P19	0.62 ± 0.10	0.42–0.82	0.033 ± 0.006
P23	0.79 ± 0.03	0.73–0.85	0.039 ± 0.008
P26	0.78 ± 0.03	0.72–0.84	0.033 ± 0.004
R17	0.75 ± 0.10	0.55–0.95	0.027 ± 0.008
U8	0.81 ± 0.06	0.69–0.93	0.027 ± 0.005
U13	0.81 ± 0.02	0.77–0.85	$0.070 \pm 0.010^*$
U17	0.86 ± 0.02	0.82–0.90	$0.043 \pm 0.008^*$
SP4	0.91 ± 0.03	0.85–0.97	0.027 ± 0.009
SS2.5	0.84 ± 0.03	0.78–0.90	0.030 ± 0.007
CPE	0.86 ± 0.02	0.82–0.90	0.027 ± 0.005
SS6	0.86 ± 0.02	0.82–0.90	0.033 ± 0.005
G3	0.82 ± 0.03	0.76–0.88	0.027 ± 0.004

* Value significantly greater than uncatalyzed rate constant ($t > 1.725$, $p < 0.05$, $df = 21$).

(α) and Chl *a*. For those stations where both primary productivity and C uptake parameters were determined ($n = 7$), neither f nor α was significantly correlated to primary productivity. Unfortunately, there were too few stations with simultaneous CO_2 and C uptake measurements ($n = 3$) for meaningful quantitative analysis.

Enzyme activities—The biomass-normalized activities of Rubisco, carbonic anhydrase, and PEPC varied substantially across the sampling stations (Table 3; Figs. 3 and 4). The greatest variability was observed for CA and PEPC (>100-fold range in activity), while Rubisco activities varied by ~50-fold across stations. Rubisco activity was positively correlated to Chl *a* ($r = 0.84$, $p < 0.001$) and negatively correlated to CO_2 concentrations ($r = -0.66$, $p = 0.01$; data not shown). Similarly, PEPC activity also showed statistically significant, albeit weak, correlations with both phytoplankton biomass and CO_2 levels. However, the correlations were opposite to those seen for Rubisco—i.e., negative correlation with Chl *a* ($r = -0.46$, $p = 0.04$) and positive correlation with CO_2 concentrations ($r = 0.60$, $p = 0.02$). PEPC activity ranged from 0.5% to 110% of Rubisco activity, with an average of $19 \pm 5\%$ (SE, $n = 26$). The PEPC/Rubisco ratio was negatively correlated to phytoplankton biomass ($r = -0.48$, $p = 0.03$; Fig. 3a) and positively correlated to CO_2 concentrations ($r = 0.62$, $p = 0.018$; Fig. 3b).

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Fig. 2. Representative isotope disequilibrium results for three stations. (a) U13, (b) U17, (c) CPE. Solid line is the least-squares curve for model fits using variable $f \text{HCO}_3^-$ and α (see text for description of these parameters). Dashed line is for a CO_2 -only uptake model ($f = 0$) with eCA expression (variable α).

Table 3. Activities of Rubisco and PEPC measured in phytoplankton samples.

Station	Rubisco activity [mg C (mg Chl a) ⁻¹ h ⁻¹]	PEPC activity [mg C (mg Chl a) ⁻¹ h ⁻¹]	PEPC:Rubisco
U6	0.81	0.17	0.21
U7	0.24	0.14	0.58
U8	0.53	0.077	0.14
U9	0.70	0.36	0.52
U10	0.64	0.12	0.19
U11	0.26	0.29	1.01
U12	0.54	0.183	0.34
U13	0.064	0.013	0.21
U14	0.20	0.006	0.03
U15	0.36	0.039	0.11
U16	0.15	0.057	0.37
U17	0.89	0.026	0.03
U20	1.5	0.033	0.02
U21	0.24	0.009	0.04
U22	0.69	0.006	0.01
U23	0.30	0.022	0.08
U24	0.99	0.061	0.06
GB5	0.094	0.002	0.02
MB2	0.075	0.003	0.05
SP4	0.12	0.011	0.10
SS2.5	0.037	0.003	0.09
CPE	0.34	0.002	0.01
SS6	0.52	0.10	0.21
G3	0.50	0.043	0.09

Total cellular carbonic anhydrase (intracellular + periplasmic) was detected in all samples. Measured activity ranged from 0.01 to 3.4 U mg protein⁻¹ (0.72 to 100 U mg Chl a ⁻¹), with an average of 1.5 U mg protein⁻¹ (39 U mg Chl a ⁻¹). Total CA activity showed a strong positive correlation with phytoplankton biomass ($r = 0.88$, $p < 0.0001$; Fig. 4a) and a striking negative correlation with CO₂ concentrations ($r = -0.92$, $p < 0.0001$; Fig. 4b).

Discussion

The results presented in this study demonstrate that direct HCO₃⁻ transport dominates C uptake by phytoplankton assemblages in both coastal and open ocean regions of the subarctic Pacific. We have recently obtained similar results in our survey of phytoplankton in the southeast Bering Sea (Martin and Tortell 2006), and evidence for substantial HCO₃⁻ transport has also been observed in the Southern Ocean (Cassar et al. 2004). Together, the available field data thus suggest that HCO₃⁻ is the dominant source of inorganic C fueling primary production in a wide range of marine environments. Moreover, HCO₃⁻ transport by in situ phytoplankton assemblages does not appear to vary with biomass, productivity, or CO₂ concentrations, suggesting that it is expressed constitutively under normal oceanic conditions.

One particularly striking result of this study is the apparent lack of eCA activity in the majority of sampled phytoplankton assemblages (10 of 15). By comparison, we were able to detect at least some eCA activity in all of the

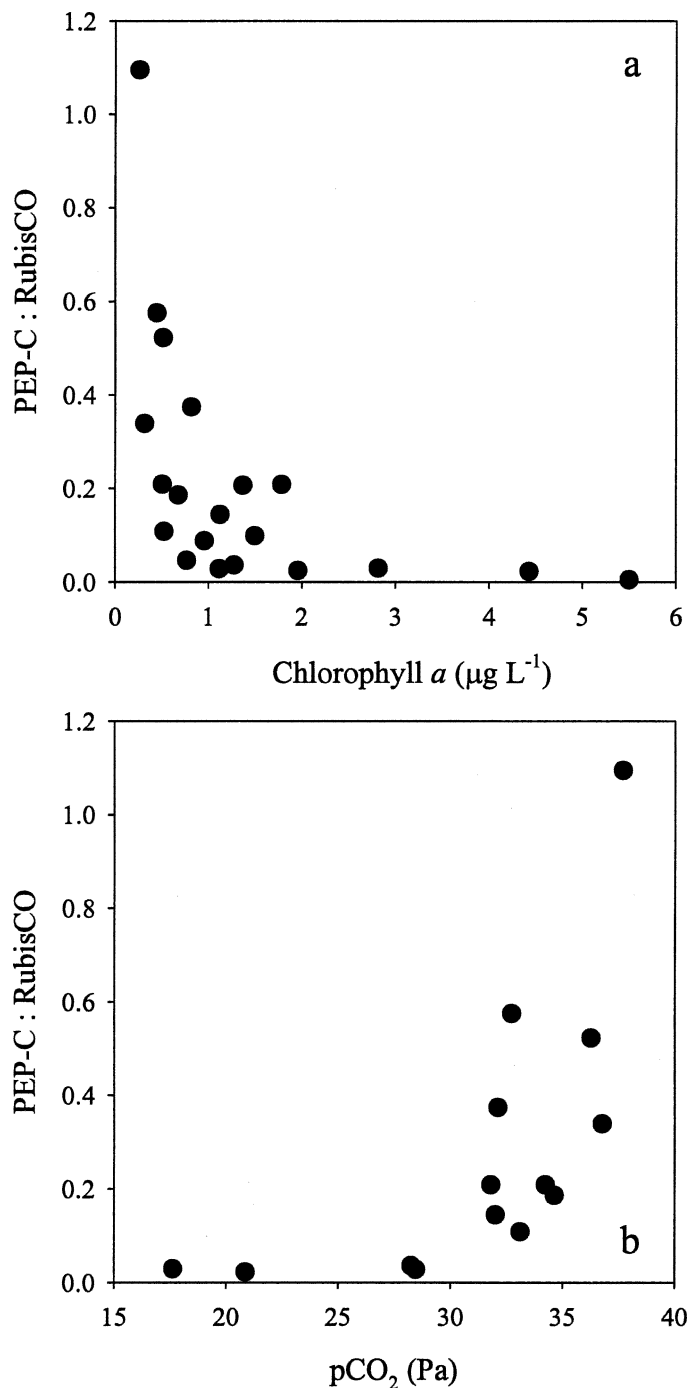


Fig. 3. Relationship between PEPC/Rubisco activity ratios and (a) phytoplankton biomass and (b) CO₂ concentrations across the sampling sites.

Bering Sea phytoplankton assemblages we examined in our recent study (Martin and Tortell 2006). At present, the reason for this difference is unclear, though it likely results at least partially from taxonomic differences among the phytoplankton assemblages. Unfortunately, phytoplankton species information is not available for our sampling sites. However, based on previous work (Mackas 1984; Booth 1988), we expect that the coastal shelf stations were dominated by a variety of diatom species, while nano-

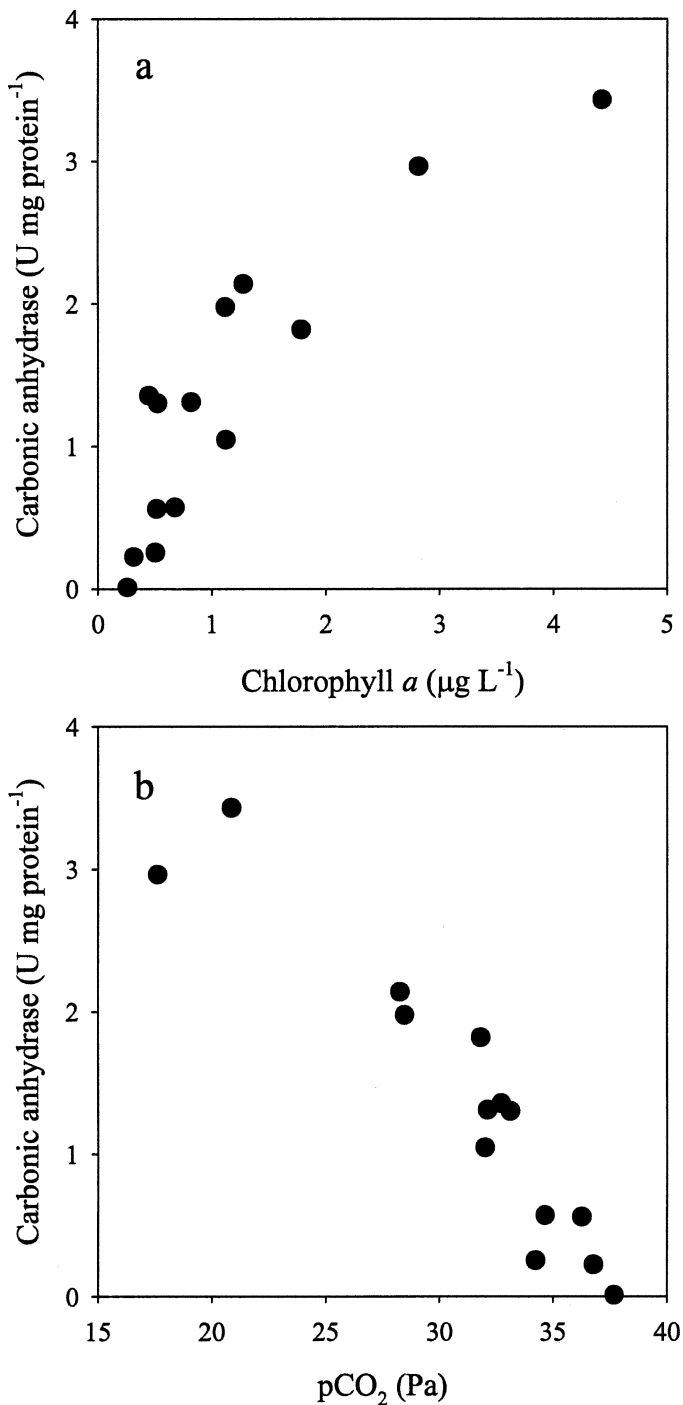


Fig. 4. As Fig. 3 but for carbonic anhydrase activity.

flagellates made a larger contribution to the phytoplankton community at open ocean sites. Laboratory studies have demonstrated a wide range of eCA activity levels among various phytoplankton taxa, with a number of species reported to lack this enzyme completely (see Colman et al. 2002; Badger 2004). Based on a survey of the literature, Colman et al. (2002) have suggested that eCA activity is restricted to species that possess an active CO₂ transport system. If this is indeed the case, low eCA activity may be

a general characteristic of phytoplankton assemblages in which C uptake is dominated by direct HCO₃⁻ transport as opposed to active CO₂ transport. Regardless of the underlying physiological explanation, our results (Table 2) call into question the significance of eCA for many phytoplankton populations in the oceans.

The transport of inorganic carbon (HCO₃⁻ and/or CO₂) across the cell membrane is only the first step in the overall process of C assimilation into photosynthetic products. Ultimately, CO₂ must be concentrated in the vicinity of Rubisco to maximize the enzyme's carboxylase activity and repress photorespiration. In many eukaryotic phytoplankton species, it is believed that HCO₃⁻ accumulates in the cytoplasm, and a chloroplast-localized CA catalyzes the subsequent dehydration of HCO₃⁻ in close proximity to Rubisco (Kaplan and Reinhold 1999; Badger 2004). While the importance of various intracellular CA isoforms has been extensively documented in laboratory studies (Sültemeyer et al. 1993; Badger 2004), very few intracellular CA measurements have been made with natural marine phytoplankton populations. Tortell et al. (2000) examined total cellular CA expression in coastal Pacific phytoplankton assemblages using Western blot analysis, potentiometric assays, and inhibitor studies. These authors were unable to detect enzyme expression and/or activity in half of their samples, and they suggested that the sequence variability of the enzyme (which limits antibody cross reactivity in Western blots) and the low sensitivity of the potentiometric assay may have been partially responsible for this. Using a more refined methodological approach, we were able to detect total CA expression (i.e., intracellular and external) in nearly all of the subarctic Pacific samples we measured in this study, with activity levels that are comparable to those measured in laboratory phytoplankton cultures (e.g., Colman and Rotatore 1995; Bhatti et al. 2002). Given the low apparent eCA activity that was present in the phytoplankton assemblages, we believe that our total CA measurements largely reflect the activity of the various intracellular isoforms.

Without localization data, our bulk activity measurements provide little insight into the physiological role of intracellular CA in the phytoplankton assemblages. Given the large contribution of direct HCO₃⁻ transport, we suggest that the enzyme functions primarily to catalyze the dehydration of HCO₃⁻ to CO₂ for fixation by Rubisco. It is unclear, at present, where this activity may be localized. If, indeed, inorganic C accumulates in the cytoplasm as HCO₃⁻, a cytoplasmic CA could potentially decrease the efficiency of the CCM by increasing CO₂ efflux from cells (Price and Badger 1989). While most studies do suggest that cellular CA is localized in the chloroplast (Sültemeyer et al. 1993; Badger 2004), a cytoplasmic CA has also been observed in at least one marine diatom (Morel et al. 2002). This enzyme might function to provide a constant CO₂ supply for subsequent transport into the chloroplast (Moroney and Mason 1991).

Although the exact biochemical role of CA in the natural phytoplankton assemblages is unclear, we did observe a striking relationship between CA activity and ambient CO₂ concentrations. This result suggests that the enzyme

plays a key role in a CO₂-regulated CCM. Such CO₂-dependent regulation of CA activity has been observed many times in laboratory cultures (*see* Sültemeyer et al. 1993; Badger 2004), but few comparable field data are available. In an early study, Berman-Frank et al. (1994) demonstrated a strong correlation between CO₂ levels and CA expression during annual blooms of the dinoflagellate *Peridinium gatunense* in Lake Kinneret. Subsequently, Tortell et al. (2000) reported elevated CA expression in marine diatom assemblages cultured with low CO₂ (10.1 Pa) in incubation experiments. Two recent studies (Tortell and Morel 2002; Martin and Tortell 2006), have reported high apparent eCA activity in low-CO₂ waters, but sample sizes were too small to allow robust quantitative analysis. By comparison, the CO₂-CA correlation observed at the 17 stations examined in this study was highly significant. To our knowledge, this is the first demonstration of such a relationship in marine waters. This result provides compelling evidence for the regulation of C acquisition across natural CO₂ gradients in the oceans.

While CA appears to play a role in C assimilation by the phytoplankton assemblages, CO₂ can also be concentrated near Rubisco via a C₄ photosynthetic pathway (Reinfelder et al. 2000). In this pathway, cytoplasmic HCO₃⁻ reacts with PEP to form oxaloacetate (and subsequently aspartate or malate), in a reaction catalyzed by PEPC. The C₄ compounds are then decarboxylated in close proximity to Rubisco, yielding a high local CO₂ concentration in the chloroplast (Hatch 1987). The C₄ mechanism does not require CA activity if HCO₃⁻ is supplied to the cytoplasm via a direct transport system. Previous laboratory and field studies have demonstrated significant PEPC activity in a number of phytoplankton species and natural assemblages (Descolas-Gros and Oriol 1992; Bentaleb et al. 1998; Fouilland et al. 2000). This β carboxylation activity was attributed, in part, to the anaplerotic role of PEPC, which replenishes intermediates for the Krebs cycle, and/or to the presence of heterotrophic (dark) C fixation (*see* Morris 1980). (These two pathways were not distinguished explicitly.) Specific evidence for the involvement of PEPC in a C₄ photosynthetic pathway came from the observation that the activity of the enzyme was enhanced under low-CO₂ conditions (Reinfelder et al. 2000), particularly when CA expression was limited by Zn deficiency.

To our knowledge, our study is the first to explicitly examine the relationship between CO₂ concentrations and PEPC activity in natural phytoplankton populations. While our measured PEPC/Rubisco activity ratios are consistent with previous field studies (Bentaleb et al. 1998; Fouilland et al. 2000), the CO₂ dependence of PEPC activity and PEPC/Rubisco ratios we observed (Fig. 3) is opposite that reported by Reinfelder et al. (2000). We found the lowest PEPC activities in high-biomass, low-CO₂ waters (Fig. 3b) where CA expression was greatest (Fig. 4b). This suggests that under low-CO₂ conditions, CA, rather than PEPC, was primarily supplying CO₂ to Rubisco from HCO₃⁻. Our results do not, however, rule out the presence of a C₄ pathway, since other β carboxylases—most notably pyruvate carboxylase—may serve the

same function as PEPC (Hatch 1987). Moreover, the measured β carboxylase activities may have been confounded by the presence of mixotrophic and heterotrophic organisms, and it is possible that the decrease in PEPC activity in low-CO₂, high-chlorophyll waters (Fig. 3) reflected a lower relative abundance of mixotrophs as previously suggested (Bentaleb et al. 1998). At present, the available field data suggest that PEPC can make a significant contribution to C fixation by marine plankton assemblages, although the exact biochemical role of this enzyme remains unclear.

Irrespective of the biochemical nature of the CCM, CO₂ must ultimately be fixed into photosynthetic products via Rubisco. This enzyme catalyzes the rate-limiting step of the Calvin cycle, and its biochemical inefficiency (low turnover rate and poor CO₂ affinity) has been well documented (Spreitzer and Salvucci 2002). Of particular importance for marine phytoplankton is the high cellular content of Rubisco and the large amount of N associated with this protein. It has been suggested that the induction of a CCM helps to maximize N-use efficiency by maintaining high CO₂ concentrations around Rubisco (Beardall et al. 1991). Yet, surprisingly little information is available on the CO₂-dependent regulation of Rubisco activity in phytoplankton. Tortell et al. (2000) observed significantly higher Rubisco expression by natural phytoplankton assemblages cultured with 15.2 Pa CO₂ compared with those maintained at 76 Pa. In contrast, a recent study with marine macroalgae reported no change in Rubisco activity in CO₂ manipulated mesocosm experiments (Israel and Hophy 2002). To our knowledge, there have been very few prior measurements of Rubisco activity levels across natural CO₂ gradients. One potential difficulty associated with such field measurements is the need to optimize assay conditions for individual isoforms of the enzyme that are present in diverse plankton assemblages (*see* MacIntyre et al. 1997). In our field survey, we found a statistically significant negative correlation between Rubisco activity and CO₂ (i.e., high activity at low CO₂). This result is consistent with the observations of Tortell et al. (2000), suggesting that enzyme activity is upregulated under low-CO₂ conditions. We note however, that Rubisco showed a much weaker CO₂ dependence than either PEPC or CA activity, suggesting that CO₂ concentrations act to regulate the various components of the CCM rather than Rubisco activity per se. The extent to which various phytoplankton regulate inorganic C uptake may be a key determinant of their responses to changing surface ocean CO₂ concentrations.

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