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Effect of growth rate and CO_2 concentration on carbon isotopic fractionation by the marine diatom *Phaeodactylum tricornutum*

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Abstract

The carbon isotopic composition ($\delta^{13}C$) of the marine diatom *Phaeodactylum tricornutum* was measured over a series of growth rates (μ) in a chemostat system in which both the $\delta^{13}C$ and the concentration of aqueous CO_2 [CO_2(aq)] were measured. CO_2(aq) ranged from 0.64 to 35 μmol kg^{-1} and growth rates from 0.5 to 1.4 d^{-1}. ϵ_p, the biological fractionation factor associated with carbon fixation, was found to be a nonlinear function of μ/CO_2(aq), contrary to the predictions of a model that assumes that CO_2 enters the cell by passive diffusion. The experimental results suggest that active uptake of bicarbonate does not account for the nonlinearity of the relationship and that inorganic carbon enters the cell as CO_2. The data are very well described by a theoretical model that assumes that *P. tricornutum* regulates the CO_2 concentration in its cytoplasm so as to minimize the energy required to concentrate CO_2 at the site of carboxylation. This is probably achieved by active uptake of CO_2 or by conversion of bicarbonate to CO_2 by an external carbonic anhydrase followed by transport of the CO_2 into the cell via either active transport or passive diffusion. Based on the model and data, $\mu/CO_2(aq) = 0.225 \times [(26.8 - \epsilon_p)/(\epsilon_p - 5.5)]$ kg d^{-1} μmol^{-1}. This equation accounts for 92% of the variance in the μ/CO_2(aq) data. The model has potential utility for estimating phytoplankton growth rates in field studies without incubations and has important implications for the estimation of ancient CO_2(aq) from the $\delta^{13}C$ of preserved organic compounds.

There is general agreement that the $^{13}C:^{12}C$ ratio of the organic carbon in plants reflects both the physiological condition of the plants and certain aspects of the environment at the time the organic matter was formed. Farquhar et al. (1982), for example, demonstrated that the combined effects of diffusion of CO_2 into a plant and isotopic discrimination by the primary carboxylating enzyme ribulose bisphosphate carboxylase oxygenase (Rubisco) would lead to approximately a linear relationship between the $\delta^{13}C$ of plant organic matter and the ration of the internal-to-external CO_2 concentrations. Fry and Wainright (1991) first recognized that both aqueous CO_2 concentrations and microalgal growth rates would influence the $\delta^{13}C$ of phytoplankton, and Rau et al. (1992) pointed out that negative correlations between the $\delta^{13}C$ of suspended particulate organic matter and concentrations of aqueous CO_2 could be explained by variations in phytoplankton demand for CO_2. Francois et al. (1993) showed that if passive diffusion dominated carbon transport into phytoplankton cells, there should exist a linear relationship between the $\delta^{13}C$ of phytoplankton organic carbon and the ratio of the carboxylation rate to external aqueous CO_2 concentration. Building on this earlier work, Goericke et al. (1994) and Laws et al. (1995) derived equations that predicted that the $\delta^{13}C$ of microalgal organic carbon should be approximately a linear function of the ratio of the microalgal growth rate (μ) to the aqueous CO_2 concentration as long as the movement of CO_2 into the cell was controlled by diffusion.

Although the biochemical characteristics of phytoplankton are in many respects similar to those of conventional C_3 higher plants (Kerby and Raven 1985), some of their gas exchange characteristics more closely resemble those of C_4 plants. Phytoplankton have high affinities for CO_2, low levels of inhibition of photosynthesis by oxygen, and low CO_2 compensation concentrations (Burns and Beardall 1987). These characteristics have suggested that they may possess a CO_2-concentrating mechanism (CCM), which increases the CO_2 concentration at the active site of Rubisco. The existence of a CCM in marine microalgae has been inferred from the fact that at low external CO_2 or inorganic carbon concentrations, the corresponding intracellular concentrations exceed the concentrations in the growth medium (Zenvirth and Kaplan 1981; Badger and Andrews 1982; Burns and Beardall 1987). Uncertainty regarding the nature of the CCM concerns (1) whether inorganic carbon is actively transported into the cell and if so whether bicarbonate or CO_2 is actively transported, (2) whether the CCM is located at the chloroplast envelope or at the plasmalemma, and (3) whether carbonic anhydrase (CA) is associated with the CCM and if so whether it is internal or external (Burns and Beardall 1987).

The existence of a CCM and the possible use of bicarbonate as a source of inorganic carbon have important implications for the interpretation of $\delta^{13}C$ values in microalgae. If inorganic carbon enters the cell primarily by active trans-

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port rather than by diffusion, then models based on diffusive transport may be misleading. Because of the relatively high concentrations of bicarbonate in seawater (~2 mM), it is generally assumed that at low CO_2 concentrations marine phytoplankton utilize bicarbonate for photosynthesis in some way. Fogel and Cifuentes (1993, p. 79), for example, commented that "At low concentrations of DIC, phytoplankton will begin to actively transport bicarbonate . . . . Internal pools of DIC will thus have enriched $\delta^{13}C$ values, as the isotope ratio of HCO_3^- is ~8‰ more positive than that of dissolved CO_2." Actually, the fractionation factor between bicarbonate and aqueous CO_2 is variable and ranges from ~8‰ at 30°C to ~12‰ at 0°C (Mook et al. 1974). Active transport of bicarbonate into the cell when aqueous CO_2 concentrations are low could lead to ^{13}C-enriched organic matter as a result of either phosphoenolpyruvate (PEP) carboxylase-mediated β-carboxylation of PEP, intracellular CA-mediated conversion to CO_2 followed by β-carboxylation of PEP or pyruvate mediated by PEP carboxykinase or pyruvate carboxylase, respectively, or intracellular CA-mediated conversion to CO_2 and subsequent carboxylation of ribulose-1,5-bisphosphate (RuBP) in the Calvin cycle. Based on short-term uptake experiments using ^{14}C-labeled substrates and measurements of enzyme activities, Morris and co-workers (Glover et al. 1975; Beardall et al. 1976) have suggested that at least under some conditions, "Photosynthesis in marine diatoms depends on an active PEPCase utilizing bicarbonate as a substrate and that a less active RuDPCase utilizes CO_2" (Beardall et al. 1976, p. 409). However, only carboxylation of RuBP would represent a net fixation of carbon, with the β-carboxylation reactions in microalgae being associated with replenishment of TCA-cycle intermediates (Goericke et al. 1994), but β-carboxylation could alter the $\delta^{13}C$ of the phytoplankton carbon. As noted by Morel and Reinfelder (1995), however, "High $\delta^{13}C$ values in phytoplankton organic matter cannot in themselves prove HCO_3^- uptake, even if many authors have made this inference."

An alternative mechanism of bicarbonate utilization would be an external CA-mediated conversion to CO_2 at the cell surface followed by diffusion or active uptake of CO_2 into the cell (Goericke et al. 1994). The existence of an external CA has been demonstrated in several marine microalgae by Burns and Beardall (1987). If such a mechanism were operative, "Isotope fractionation by carbonic anhydrase, which for the catalysed conversion of HCO_3^- to CO_2 is 10.1‰ . . . would largely cancel the initial difference in $\delta^{13}C$ . . . between HCO_3^- and CO_2. The $\delta^{13}C$ signal resulting from carbonic anhydrase-mediated HCO_3^- uptake and CO_2 uptake would essentially be indistinguishable" (Riebesell and Wolf-Gladrow 1995).

Notably missing from the debate about inorganic carbon utilization and phytoplankton $\delta^{13}C$ values have been many data from phytoplankton cultures grown under conditions in which the chemistry of the growth medium and the physiological condition of the phytoplankton were well defined. Falkowski's (1991) batch culture work with 13 species of marine phytoplankton was an important first step, but since the growth rates of the algae were not reported, it has been impossible to use the data to test models such as those of Goericke et al. (1994) and Laws et al. (1995). Thompson and Calvert (1994, 1995) recently reported results of carbon isotope fractionation studies on batch cultures of *Thalassiosira pseudonana* and *Emiliania huxleyi*, but an error was made in the application of the Rayleigh distillation model (Mariotti et al. 1981) to CO_2 uptake (Laws et al. 1998). The work described here is an extension of an earlier study of the marine diatom *Phaeodactylum tricornutum* (Laws et al. 1995) in which continuous-culture methods have been used to control growth rates and to create steady-state conditions in which both the concentrations and isotopic compositions of the inorganic carbon in the growth medium were well defined.

The literature concerning the ability of *P. tricornutum* to utilize bicarbonate and concentrate CO_2 is contradictory. Patel and Merrett (1986) and Colman and Rotatore (1995) concluded that all CA activity in *P. tricornutum* was intracellular, whereas Burns and Beardall (1987) and Dionisio-Sese and Miyachi (1992) reported that a substantial percentage of the CA activity was external. Patel and Merrett (1986) and Dixon and Merrett (1988) found that internal dissolved inorganic carbon (DIC) concentrations in *P. tricornutum* were <20% of external DIC concentrations even at external DIC concentrations as low as 50 μM. However, Burns and Beardall (1987) and Colman and Rotatore (1995) reported internal DIC concentrations that were 2–5 times external DIC concentrations when the latter were ≤1.1 mM, and Burns and Beardall (1987) estimated internal CO_2 concentrations to be ~5–10 times external aqueous CO_2 concentrations when the latter were ≤6 μM. Dixon and Merrett (1988, p. 47) concluded that their results were "in agreement with bicarbonate transport across the plasmalemma," whereas Burns and Beardall (1987, p. 84) concluded that "Despite apparent utilization of HCO_3^- from the bulk medium, CO_2 is the inorganic carbon species crossing the plasmalemma."

The earlier study of Laws et al. (1995) involved *P. tricornutum* cultures grown at aqueous CO_2 concentrations of 10–35 μmol kg^{-1}, comparable to or higher than the values of 10–20 μmol kg^{-1} typically found in seawater (Rau et al. 1992; Francois et al. 1993). That work revealed that ϵ_p, approximately the difference between the $\delta^{13}C$ of the aqueous CO_2 and the phytoplankton carbon, was well described by a linear function of the ratio of growth rate to aqueous CO_2 concentration [$\mu/CO_2(aq)$], in accord with the predictions of a model that assumed diffusive transport of CO_2 into the cell. The present study reports work carried out at aqueous CO_2 concentrations as low as 0.64 μmol kg^{-1}, well within the range of aqueous CO_2 concentrations where Burns and Beardall (1987) found evidence of a CCM. A principal motivation for the work was to determine whether the simple linear relationship between ϵ_p and $\mu/CO_2(aq)$ would extend to low $CO_2(aq)$, or whether the model would have to be modified in some way to account for the effects of the CCM and bicarbonate utilization.

Materials and methods

The laboratory methods were identical to those described by Laws et al. (1995) and will be summarized here only briefly. *P. tricornutum* Bohlin (clone CCMP1327, Center for

Table 1. Results of *Phaeodactylum tricornutum* chemostat studies.

CO_2(aq) (μmol kg^{-1})	pH	DIC (mmol kg^{-1})	μ (d^{-1})	μ/CO_2 (kg d^{-1} μmol^{-1})	ϵ_p	$\delta^{13}C_{DIC}$	$\delta^{13}C_{CO_2}$	$\delta^{13}C_p$	C/cell (pg cell^{-1})
					(‰)				
0.64	8.94	1.39	1.38	2.163	7.42	−0.67	−8.85	−16.15	4.95
0.92	8.82	1.36	1.04	1.134	11.08	−1.48	−9.75	−20.60	—
0.98	8.82	1.45	0.50	0.509	10.65	−1.47	−6.83	−17.30	5.82
2.36	8.58	1.67	0.75	0.318	11.83	−1.07	−9.51	−21.09	6.99
2.93	8.51	1.68	0.75	0.256	16.76	−1.99	−10.47	−26.79	6.21
3.56	8.46	1.76	0.50	0.140	20.63	−1.18	−9.70	−29.73	7.24
6.49	8.26	1.86	0.50	0.077	19.85	−5.21	−13.79	−32.99	7.94
10.27	8.11	2.01	1.40	0.136	18.36	−8.84	−17.43	−35.14	11.00
10.72	8.10	2.02	1.25	0.117	18.94	−8.97	−17.56	−35.82	9.15
11.98	8.06	2.05	1.00	0.083	20.58	−9.72	−18.31	−38.10	7.53
15.61	7.97	2.10	0.75	0.048	22.04	−12.18	−20.77	−41.88	8.20
22.23	7.83	2.17	0.50	0.022	24.35	−15.95	−24.51	−47.70	9.57
34.71	7.67	2.27	0.50	0.014	25.72	−7.17	−15.80	−40.47	6.87

Culture of Marine Phytoplankton, West Boothbay Harbor, Maine) was grown in a nitrate-limited chemostat system at a temperature of 22.0 ± 0.1°C and salinity of 33‰. Light was provided by a bank of daylight fluorescent lamps at an intensity of ~21.6 mol quanta m^{-2} d^{-1} (400–700 nm radiation). The concentration of nitrate in the growth medium was 100 μM. The partial pressure of CO_2 in the gas used to aerate the growth chamber was controlled using mass-flow controllers to adjust the flow rates of tank CO_2 (2.06% CO_2 in air) and CO_2-free air. Sampling for isotopic analysis of the particulate carbon in the growth chamber was not begun until the culture had completed at least four doublings at a given growth rate and the $\delta^{13}C$ of the DIC in the growth chamber had stabilized to ±0.1‰ from day to day.

Samples were taken on a daily basis for determination of the concentration and isotopic composition of the DIC. DIC and $\delta^{13}C_{DIC}$ were determined using a system modified after Kroopnick (1985). The concentration of CO_2(aq) was determined from concentrations of DIC, phosphate, and silicate, as well as total alkalinity (Roy et al. 1993; Millero 1995). Phosphate and silicate analyses were performed using the colorimetric techniques described in Strickland and Parsons (1972) on a Technicon Auto Analyzer II. Total alkalinity was determined by the Gran method using a computer-controlled titration. The isotopic composition of CO_2(aq) was calculated using the mass balance equation

$$\epsilon_{da} = \epsilon_{ba} X_b + \epsilon_{ca} X_c, \quad (1)$$

where the subscripts a, b, c, and d refer to CO_2(aq), bicarbonate, carbonate, and DIC, respectively, $\epsilon_{jk} = \{(\delta^{13}C_j - \delta^{13}C_k) \div [1 + (\delta^{13}C_k/1{,}000)]\}$ and X_b and X_c are the mole fractions of bicarbonate and carbonate in the DIC calculated using equations 41 and 42 in Millero (1995) for the first and second apparent dissociation constants of carbonic acid. ϵ_{ba} and ϵ_{ca} were set equal to 8.95 and 7.45‰ based on equations 13, 14, and 18 in Deines et al. (1974).

Samples of *P. tricornutum* for carbon isotopic analysis were filtered onto precombusted (500°C for at least 4 h) Whatman GF/C glass-fiber filters. The filters were wrapped in precombusted aluminum foil and immediately placed in and stored under liquid nitrogen until analysis. Frozen samples were placed in precombusted quartz tubes, vacuum dried, cupric oxide was added, the tubes were sealed, and the samples were combusted at 680°C for at least 8 h (Wedeking et al. 1983). Isotope abundances were measured on cryogenically purified CO_2 using either a Finnigan MAT 252 or Delta-S mass spectrometer (Santrock et al. 1985).

Particulate carbon concentrations were determined by two methods: manometrically from the amount of CO_2 produced when the samples for particulate carbon isotopic analysis were combusted and from 50-ml aliquots from the growth chamber collected on precombusted GF/C filters analyzed on a Perkin-Elmer model 2400 CHN elemental analyzer. Agreement between the two methods was quite good (r^2 = 0.96). Cell counts were measured with a Celloscope particle counter. Although in the studies reported here most *P. tricornutum* cells were solitary, microscopic analysis revealed the presence of some short chains of two to five cells. Because the particle counter recorded short chains as single particles, cell counts were calculated from particle count results by microscopic analysis of the relative abundance of solitary cells and short chains. The correction factor increased the counts by 10–65%. Carbon cell quotas were then calculated from the ratio of particulate carbon and cell counts.

Results

The results are shown in Table 1 and Fig. 1. Values of ϵ_p were calculated from the equation

$$\epsilon_p = \frac{\delta^{13}C_a - \delta^{13}C_p}{1 + (\delta^{13}C_p/1{,}000)} \quad (2)$$

(Freeman and Hayes 1992; Goericke et al. 1994), where $\delta^{13}C_a$ is the $\delta^{13}C$ of the aqueous CO_2 and $\delta^{13}C_p$ is the $\delta^{13}C$ of the phytoplankton carbon.

There is a highly significant negative correlation between ϵ_p and μ/CO_2(aq), with the Spearman rank correlation coefficient being −0.923 ($P < 0.01$). However, a simple linear equation treating ϵ_p as the independent variable gives a rather poor fit to the μ/CO_2(aq) data (R^2 = 0.59). The goodness-

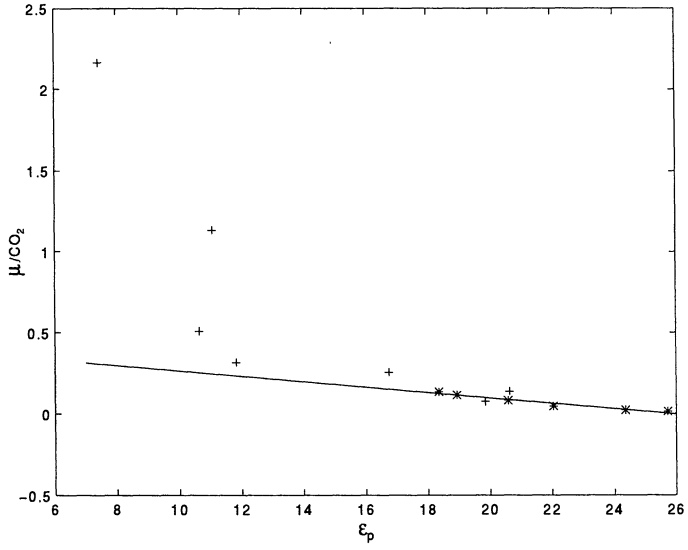

Fig. 1. Relationship between μ/CO_2(aq) (kg d^{-1} μmol^{-1}) and ϵ_p for *P. tricornutum*. The straight line is the least-squares equation fit to the data collected at CO_2(aq) > 10 μmol kg^{-1} (*). Plus signs indicate data collected at CO_2(aq) < 7 μmol kg^{-1}.

of-fit is significantly improved with the addition of a quadratic term to the polynomial (R^2 = 0.81). All terms in the quadratic polynomial are statistically significant at $P < 0.01$ by a *t*-test. A noteworthy aspect of the data is the fact that the five highest μ/CO_2(aq) values, all of which were obtained at CO_2(aq) concentrations of <4 μmol kg^{-1}, all lie above the linear regression line fit to the data obtained at CO_2(aq) concentrations of 10–35 μmol kg^{-1}. We conclude that there is evidence of significant nonlinearity in the relationship between μ/CO_2(aq) and ϵ_p when the range of μ/CO_2(aq) is extended by including results from CO_2(aq) concentrations below ~4 μmol kg^{-1}. Hence, we must reject the hypothesis that the underlying relationship between ϵ_p and μ/CO_2(aq) is linear over the CO_2(aq) concentration range 0.64–35 μmol kg^{-1}.

To gain some insight into the cause of the nonlinearity, we consider the model of carbon uptake and assimilation presented by Francois et al. (1993). Assuming that isotope discrimination effects due to respiration and photorespiration are negligible (Laws et al. 1995), Francois et al. (1993) showed that ϵ_p should closely follow the equation.

$$\epsilon_p = \epsilon_1 + f(\epsilon_2 - \epsilon_{-1}), \tag{3}$$

where ϵ_1, ϵ_{-1}, and ϵ_2 are the isotopic discriminations associated with whatever process brings inorganic carbon through the plasmalemma into the cell, diffusion back into the surrounding water, and enzymatic carboxylation to produce phytoplankton biomass, respectively, and f is the fraction of the inorganic carbon taken up by the cell that diffuses back into the water. Eq. 3 can be derived from nothing more than mass balance considerations and in particular makes no assumptions about the mechanism by which inorganic carbon enters the cell. For eukaryotic microalgae it is customary to assume that the rate of diffusion back into the surrounding water is equal to $k_{-1}C_i$, where k_{-1} is the permeability of the plasmalemma to CO_2 and C_i is the concentration of CO_2 in the cytoplasm. Assuming that C_i is constant, the rate of inorganic carbon uptake per cell from the water must equal the carbon utilized for biomass production plus the carbon that diffuses back into the water, i.e.

$$\text{uptake per cell} = \mu C + k_{-1}C_i, \tag{4}$$

where C is the carbon cell quota of the microalga. Hence, $f = k_{-1}C_i/(\mu C + k_{-1}C_i)$ and

$$\epsilon_p = \epsilon_1 + \frac{\epsilon_2 - \epsilon_{-1}}{1 + (\mu C/k_{-1}C_i)}. \tag{5}$$

Eq. 5 still makes no assumptions about the mechanism by which inorganic carbon enters the cell. Based on this model, the maximum possible value of ϵ_p is $\epsilon_2 + \epsilon_1 - \epsilon_{-1}$. ϵ_{-1} is generally assumed to equal ~0.7‰, the isotope effect associated with diffusion of inorganic carbon in fresh water at 25°C (O'Leary 1984). If inorganic carbon enters the cell via diffusion of CO_2, then it is reasonable to assume that $\epsilon_1 = \epsilon_{-1}$, in which case the maximum value of ϵ_p is ϵ_2. ϵ_2 is the combined fractionation due to Rubisco and β-carboxylations, and its likely value is 25–28‰ (Goericke et al. 1994). It is not known whether isotopic discrimination occurs during active uptake of inorganic carbon, but it is generally assumed that the effect is negligible (Kerby and Raven 1985; Berry 1989). The implication is that the maximum value of ϵ_p is ~25–28‰, regardless of whether inorganic carbon enters the cell by diffusion or a combination of diffusion and active transport.

Had we assumed that inorganic carbon entered the cell in the form of bicarbonate rather than of CO_2, then $\delta^{13}C_a$ in Eq. 2 would have been replaced by $\delta^{13}C_b$, and the calculated values of ϵ_p would have increased by ~9‰ (Deines et al. 1974; Mook et al. 1974). Six of the 12 ϵ_p values would have exceeded 28‰, and 9 of the 12 would have exceeded 25‰. The fourth highest ϵ_p value (20.6‰) is associated with a CO_2(aq) of 3.6 μmol kg^{-1}, and the three lowest ϵ_p values are all associated with CO_2(aq) of <2.4 μmol kg^{-1} (Table 1). We conclude from this analysis that active transport of bicarbonate into the cell was not a significant source of inorganic carbon for *P. tricornutum* at CO_2(aq) concentrations > ~3 μmol kg^{-1}. It is apparent from Fig. 1 that at the lowest CO_2(aq) concentrations the phytoplankton carbon was isotopically lighter than would have been predicted from extrapolation of the trend in ϵ_p values at higher CO_2(aq) concentrations. This is an improbable result if the cells had switched to bicarbonate uptake at low CO_2(aq) concentrations, since the bicarbonate was isotopically heavy compared to the CO_2(aq) by ~9‰. We conclude from this analysis that bicarbonate uptake was not a significant source of inorganic carbon in any of these experiments and does not account for the nonlinearity of the data in Fig. 1.

The implication of this conclusion is that the nonlinearity of the data in Fig. 1 is the result of CO_2 entering the cell by some mechanism other than passive diffusion. To gain some insight into the nature of the process, we consider the possibility that inorganic carbon enters the cell by both active transport and diffusion and, having entered the cell, is actively transported from the cytoplasm to the site of carboxylation. We can assume that the energetic costs associated

with these active transport steps are proportional to the associated fluxes of inorganic carbon and are linearly related to the corresponding concentration gradients. The energetic cost E is therefore given by the equation

$$E = F_1[A_1 + B_1(C_i - C_e)] + F_2[A_2 + B_2(C_c - C_i)], \quad (6)$$

where F_1 is proportional to the flux of inorganic carbon due to active transport across the plasmalemma; F_2 is proportional to the corresponding flux from the cytoplasm to the site of carboxylation; C_e, C_i, and C_c are the concentrations of inorganic carbon in the bulk medium, immediately interior to the plasmalemma, and at the site of carboxylation, respectively; and A_1, A_2, B_1, and B_2 are constants. A_1 is the energetic cost of transporting one unit of CO_2 across the plasmalemma when $C_i = C_e$, and B_1 is the rate of change of that energetic cost per unit change in $C_i - C_e$. A_2 and B_2 have similar meanings with respect to the transport of CO_2 from a location immediately interior to the plasmalemma to the site of carboxylation. We assume that CO_2 diffuses back and forth across the plasmalemma at a net rate (F_d) proportional to the concentration gradient across the plasmalemma. If C_i is constant, then $F_1 - F_2 = F_d = k_{-1}(C_i - C_e)$. It follows that $F_1 = F_2 + k_{-1}(C_i - C_e)$. It is now reasonable to equate F_2 to the photosynthetic rate of the cell, μC. Hence, $F_1 = \mu C + k_{-1}(C_i - C_e)$. Substituting μC for F_2 and $\mu C + k_{-1}(C_i - C_e)$ for F_1 in Eq. 6, we obtain

$$E = k_{-1}(C_i - C_e)[A_1 + B_1(C_i - C_e)] + \mu C[A_1 + A_2 + B_1(C_i - C_e) + B_2(C_c - C_i)]. \quad (7)$$

We now assume that through active transport the cell regulates C_i so that this energetic cost is minimized. Differentiating the right-hand side of Eq. 7 with respect to C_i we obtain

$$\frac{dE}{dC_i} = 2k_{-1}B_1(C_i - C_e) + k_{-1}A_1 - \mu C(B_2 - B_1). \quad (8)$$

Setting this derivative equal to zero and solving for C_i gives

$$C_i = C_e + \frac{\mu C(B_2 - B_1) - k_{-1}A_1}{2k_{-1}B_1}. \quad (9)$$

Because it follows immediately from Eq. 8 that $(d^2E/dC_i^2) = 2k_{-1}B_1 > 0$, it is clear that the value of C_i calculated from the right-hand side of Eq. 9 minimizes E.

By rearranging Eq. 5 we find that

$$C_i = \frac{\mu C}{k_{-1}}\left(\frac{\epsilon_p - \epsilon_1}{\epsilon_2 - \epsilon_{-1} + \epsilon_1 - \epsilon_p}\right). \quad (10)$$

Substituting the right-hand side of Eq. 10 for C_i in Eq. 9 and rearranging gives

$$C_e = C_o + \frac{\mu C}{k_{-1}}\left(\frac{\epsilon_p - \epsilon_1}{\epsilon_2' - \epsilon_p} - \beta\right), \quad (11)$$

where $\epsilon_2' = \epsilon_2 - \epsilon_{-1} + \epsilon_1$, $C_o = (A_1/2B_1)$ and $\beta = (B_2 - B_1)/2B_1$. Rearranging Eq. 11 gives

$$\frac{\mu}{C_e - C_o} = \frac{k_{-1}}{C(1 + \beta)}\left(\frac{\epsilon_2' - \epsilon_p}{\epsilon_p - \epsilon_1'}\right), \quad (12)$$

where $\epsilon_1' = \epsilon_1 + [\beta/(1 + \beta)] \times (\epsilon_2' - \epsilon_1)$. Because both ϵ_1 and ϵ_{-1} are expected to be small, it is reasonable to anticipate that ϵ_2' will be comparable to ϵ_2. However, because ϵ_2 is thought to be much larger than ϵ_1, it is possible that ϵ_1' will differ substantially from ϵ_1 if β is not close to zero. The implication of the model is that $[\mu/(C_e - C_o)] \times [(\epsilon_p - \epsilon_1')/(\epsilon_2' - \epsilon_p)]$ should be a constant equal to $k_{-1}/[C(1 + \beta)]$. To check this hypothesis, we carried out a least-squares analysis using the data in Table 1 and choosing C_o, ϵ_1', and ϵ_2' so as to minimize the C.V. (SD divided by the absolute value of the mean) of $[\mu/(C_e - C_o)] \times [(\epsilon_p - \epsilon_1')/(\epsilon_2' - \epsilon_p)]$. The results of this exercise gave $\epsilon_1' = 5.5 \pm 0.55‰$, $\epsilon_2' = 26.8 \pm 3.3‰$, and $C_o = 0 \pm 0.27$ μmol kg^{-1}. The mean value of $[\mu/(C_e - C_o)] \times [(\epsilon_p - \epsilon_1')/(\epsilon_2' - \epsilon_p)]$ was 0.225 with a C.V. of 35%. The equation describing our results therefore becomes

$$\frac{\mu}{C_e} = 0.225\left(\frac{26.8 - \epsilon_p}{\epsilon_p - 5.5}\right). \quad (13)$$

A graph of this relationship is shown in Fig. 2. Fig. 2A is directly comparable to Fig. 1. The nonlinear model accounts for 92% of the variance in μ/C_e and clearly gives a far better

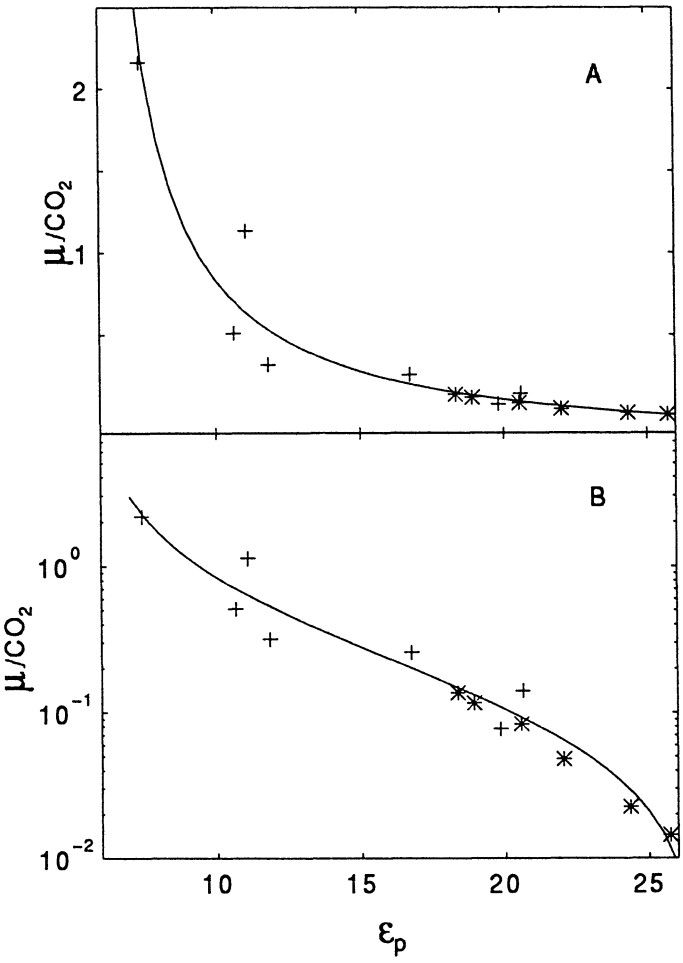

Fig. 2. Same data as shown in Fig. 1. The curved line corresponds to the equation $\mu/CO_2(aq) = 0.225 \times [(26.8 - \epsilon_p)/(\epsilon_p - 5.5)]$ kg d^{-1} μmol^{-1}. In Fig. 2A, $\mu/CO_2(aq)$ is plotted on a linear scale and in Fig. 2B on a logarithmic scale.

fit to the data than does the linear model in Fig. 1. Fig. 2B makes it clear that the model accurately describes the results at both high and low μ/C_e.

Discussion

The results of this study leave little doubt that for *P. tricornutum* the relationship between ϵ_p and μ/CO_2(aq) is nonlinear over the μ/CO_2(aq) range 0–2.5 kg d^{-1} μmol^{-1}. If *P. tricornutum* obtained all its carbon by simple diffusion from the bulk medium, ϵ_p should be well approximated by a linear function of μ/CO_2(aq) (Goericke et al. 1994; Laws et al. 1995). The implication is that *P. tricornutum* is able to obtain inorganic carbon from seawater by some means other than simple diffusion from the bulk medium.

Based on an analysis of data obtained at CO_2(aq) >10 $\mu mol\ kg^{-1}$, Laws et al. (1995) previously concluded that the relationship between ϵ_p and μ/CO_2(aq) for *P. tricornutum* was linear and hence consistent with uptake via passive diffusion from the medium. In fact a linear fit to the data at CO_2(aq) >10 $\mu mol\ kg^{-1}$ is quite good ($r^2 = 0.957$), and the second-order coefficient in a quadratic model is not significant at $P = 0.05$ by a *t*-test. Therefore, by the usual statistical criterion there is no basis for rejecting the hypothesis that the relationship is linear if only data at CO_2(aq) >10 $\mu mol\ kg^{-1}$ are considered. The quadratic coefficient, however, is significant at $P = 0.064$, and hence there is a suggestion of nonlinearity. The nonlinearity becomes apparent when the range of μ/CO_2(aq) is expanded by including data at CO_2(aq) <7 $\mu mol\ kg^{-1}$ (Fig. 1), and the equation $\mu/C_e = 0.225 \times [(26.8 - \epsilon_p)/(\epsilon_p - 5.5)]$ gives almost as good a fit to the data at CO_2(aq) >10 $\mu mol\ kg^{-1}$ ($R^2 = 0.939$) as does the linear model. In fact, any well-behaved function $f(x)$ can be well approximated as a linear function of x over a sufficiently small range of x, and the linearity apparent in Fig. 1 between ϵ_p and μ/CO_2(aq) for μ/CO_2(aq) <0.3 kg d^{-1} μmol^{-1} reflects this fact. While the relationship between ϵ_p and μ/CO_2(aq) may therefore be nonlinear, the implication of Fig. 1 is that carbon isotopic fractionation by *P. tricornutum* can be well approximated by a linear model for $0 < \mu/CO_2$(aq) <0.3 kg d^{-} μmol^{-1}, a range that includes most growth-rate and CO_2(aq) combinations found in seawater.

Several lines of evidence suggest that active transport of bicarbonate into the cell does not account for the nonlinear nature of the complete dataset. First, based on the theoretical model of Francois et al. (1993) the maximum value of ϵ_p should be comparable to ϵ_2. Francois et al.'s model (Eq. 5) makes no assumptions about the mechanism by which inorganic carbon enters the cell. Assuming that $\epsilon_1 - \epsilon_{-1} \cong 0‰$ (Kerby and Raven 1985; Berry 1989; Hayes 1993), the parameters of our model imply that $\epsilon_2' = \epsilon_2 = 26.8‰$, within the range of probable values for ϵ_2 (25–28‰) suggested by Goericke et al. (1994) and similar to Hayes' (1993) estimate of ~27‰. Thus our calculated value of ϵ_2' seems reasonable. If bicarbonate were the source of the inorganic carbon entering the cell, our calculated ϵ_p values would have to be increased by ~9‰, and half would exceed 28‰, the approximate upper bound on ϵ_2. Second, if the nonlinear nature of the relationship in Fig. 1 were due to active uptake of bicarbonate at high μ/CO_2(aq) values, the curvature of the relationship would be negative rather than positive, because the phytoplankton carbon would become more enriched in ^{13}C than would be predicted from a tangent drawn to the curve at low μ/CO_2(aq). In fact, the curvature is positive. Finally, if we were underestimating ϵ_p by 9‰ by inappropriately assuming CO_2(aq) rather than bicarbonate to be the source of inorganic carbon taken up by the cells, it is quite possible that our calculated ϵ_p values would become negative as μ/CO_2(aq) becomes large (Hayes 1993). In fact, all of our ϵ_p values are positive, and the equation fit to the data predicts that ϵ_p will exceed 5.5‰ for all finite μ/CO_2(aq).

The evidence therefore strongly suggests that active uptake of bicarbonate does not account for the nonlinearity of the data in Fig. 1. Before dismissing this possibility, however, it seems appropriate to ask whether the CO_2 in the bulk medium could have supplied all the CO_2 needed by *P. tricornutum*. Using equation 6 in Johnson (1982) and the data in Table 1, we can calculate the uncatalyzed rate of formation of CO_2(aq) from the dehydration and dissociation of bicarbonate and compare that rate to the rate of net photosynthetic uptake by the algae, μC. The result of this calculation shows that the uncatalyzed rate of formation of CO_2(aq) from bicarbonate was at least 12 times the photosynthetic rate of the algae in the growth chamber. Hence, the CO_2 in the bulk medium should not have been a limiting factor. A second concern is whether diffusion of CO_2 through the boundary layer around the cell could have supplied the CO_2 needs of *P. tricornutum*. The models of Pasciak and Gavis (1975) and Riebesell et al. (1993) provide a means of answering this question. Our particulate carbon and cell count results show that *P. tricornutum* has a carbon cell content of 7.6 ± 1.7 pg $cell^{-1}$. Based on the empirical relationship between cell volume and carbon content reported by Montagnes et al. (1994), the volume of *P. tricornutum* is ~72.5 μm^3. Its equivalent spherical radius is therefore ~2.6 μm. Microscopic analysis, however, reveals that its surface-to-volume ratio is about 2.4 μm^{-1}, equivalent to that of a sphere with a radius of 1.25 μm. The high surface-to-volume ratio reflects the fact that *P. tricornutum* is shaped more like an elongated cylinder than a sphere, the length of the cylinder being 14.4 μm and the diameter ~1.7 μm. Its geometry can therefore be well approximated by assuming each cell to be a prolate spheroid with an eccentricity e of $[1 - (1.7^2/14.4^2)]^{1/2} = 0.993$ (Pasciak and Gavis 1975). Using Eq. 3 in Riebesell et al. (1993) with a shape factor correction for prolate spheroids given by equation 14 in Pasciak and Gavis (1975) and ignoring any conversion of bicarbonate to CO_2 within the boundary layer, we estimate a CO_2 flux to the cell surface equal to 4.38×10^{-9} $(C_e - C_s)$ m^3 d^{-1} $cell^{-1}$, where C_s is the CO_2(aq) concentration at the cell surface and C_e is the equilibrium CO_2(aq) concentration in the bulk medium. Noting that for carbon 1 $\mu M = 12 \times 10^9$ pg m^{-3}, the C_s/C_e ratio required to support a given growth rate μ is therefore given by the equation

$$\frac{C_s}{C_e} = 1 - (7.6 \text{ pg cell}^{-1}) \div \{[4.38 \times 10^{-9} \text{ m}^3 \text{ d}^{-1} \text{ cell}^{-1}] \times [12 \times 10^9 \text{ pg m}^{-3}(\mu\text{M})^{-1}]\}\frac{\mu}{C_e}, \quad (14)$$

where the units of μ/C_e are d^{-1} μM^{-1}. By far the largest μ/C_e in Table 1 is 2.16 kg μmol^{-1} d^{-1} = 2.11 d^{-1} μM^{-1}. Inserting this value of μ/C_e into the right-hand side of Eq. 14 gives C_s/C_e = 0.695. This is the smallest C_s/C_e ratio required to support any of the growth rates in Table 1. The implication of this calculation is that diffusion of CO_2 from the bulk medium through the boundary layer around the cell was probably not limiting to *P. tricornutum* for any of the μ/C_e ratios we examined. A corollary conclusion is that C_s/C_e was close to 1 in almost all cases.

The implications of Eq. 9 are qualitatively consistent with the observations of Patel and Merrett (1986) and Burns and Beardall (1987). Although the two studies disagree as to whether *P. tricornutum* can accumulate inorganic carbon relative to the external medium, both studies show a positive correlation between the internal and external concentrations of inorganic carbon or CO_2, respectively. The data of Burns and Beardall (1987) show some curvature. The data of Patel and Merrett (1986) are distinctly linear.

With C_o and β as previously defined, Eq. 9 can be written in the form

$$C_i = C_e - C_o + \beta\frac{\mu C}{k_{-1}}. \quad (15)$$

Two important caveats to this equation are that $F_1 = \mu C + k_{-1}(C_i - C_e) \geq 0$ and that $C_i \geq 0$. The first of these conditions simply states that any active transport of inorganic carbon must be into rather than out of the cell. Mathematically, the first condition requires that $C_i \geq C_e - (\mu C/k_{-1})$. The constraint on Eq. 15 is therefore that $C_e - C_o + \beta(\mu C/k_{-1}) \geq C_e - (\mu C/k_{-1})$. Rearranging this inequality gives the constraint equation

$$\mu \geq \frac{C_o k_{-1}}{C(1 + \beta)}. \quad (16)$$

This condition is automatically satisfied if $C_o = 0$, which is the value of C_o that gives the best fit to our data. However, because the SD of C_o is 0.27 $\mu mol\ kg^{-1}$, it seems appropriate to explore the implications of $C_o \neq 0$. A negative C_o would make no sense biologically, but $C_o > 0$ would imply no active transport of inorganic carbon into the cell at growth rates less than $C_o\ k_{-1}/[C(1 + \beta)]$ as long as the cell's requirements for inorganic carbon could be met by diffusion of CO_2. The requirement that $C_i \geq 0$ imposes a limit on how fast the cell can obtain CO_2 via diffusion. The maximum rate, corresponding to $C_i = 0$, is $C_e k_{-1}/C$. Hence, it is to be expected that the cell will obtain its inorganic carbon entirely by diffusion of CO_2 as long as μ is less than or equal to the smaller of $C_o k_{-1}/[C(1 + \beta)]$ and $C_e k_{-1}/C$. Furthermore, even at growth rates greater than $C_o k_{-1}/[C(1 + \beta)]$, it is clear from Eq. 15 that there will be a net diffusion of CO_2 into the cell (i.e. $C_i < C_e$) as long as $\mu < (C_o k_{-1}/\beta C)$.

Assuming that ϵ_1 is small compared to ϵ_2', our least-squares estimate of ϵ_1' should be $\sim(\beta/1 + \beta)\epsilon_2'$. With ϵ_1' = 5.5 and ϵ_2' = 26.8, we conclude that β = 0.26. As previously noted, C for *P. tricornutum* is ~7.6 pg $cell^{-1}$ (=0.63 × 10^{-6} $\mu mol\ cell^{-1}$). Because our least-squares analysis indicates that $k_{-1}/[C(1 + \beta)]$ = 0.225, we conclude that k_{-1} = (0.63 × 10^{-6})(1.26)(0.225) = 1.79 × 10^{-7} liters d^{-1}. Dividing this figure by the surface area of *P. tricornutum* (174 μm^2) gives the permeability in more conventional units of length per unit time, 1.03 m d^{-1}. This figure is about 8 times smaller than the permeability postulated by Rau et al. (1996). Most of the discrepancy can be traced to Rau et al.'s (1996) assumption that phytoplankton cells are spherical and that all CO_2 enters the cells by passive diffusion. For example, *P. tricornutum* has a surface-to-volume ratio over twice that of a spherical cell with the same volume.

Adding two SDs to obtain an approximate upper bound to C_o, we conclude that C_o for *P. tricornutum* could be as great as 0.54 $\mu mol\ kg^{-1}$ and hence that $C_o k_{-1}/[C(1 + \beta)]$ could be as great as 0.54 × 0.225 (=0.122 d^{-1}). Likewise, $C_o k_{-1}/\beta C$ could be as great as 0.54 × 0.225 × (1 + β/β = 0.54 × 0.225 × (1.26/0.26) (=0.59 d^{-1}). A growth rate of 0.122 d^{-1} is lower than any of the growth rates used in our study, and hence it seems unlikely that passive diffusion of CO_2 accounted for all the inorganic carbon taken up by *P. tricornutum* in any of our experiments. This conclusion is consistent with the fact that the equation μ/C_e = 0.225 × [(26.8 − ϵ_p)/(ϵ_p − 5.5)] gives virtually the same goodness-of-fit to the data collected at C_e >10 $\mu mol\ kg^{-1}$ as a linear model derived from the assumption that CO_2 enters the cell entirely by passive diffusion. However, 5 of our 13 experiments were carried out at growth rates of 0.5 d^{-1}. The implication of our model is that $C_i < C_e$ for $\mu < 0.59\ d^{-1}$, and hence some CO_2 would have entered the cells by passive diffusion at growth rates of 0.5 d^{-1} if we assume C_o as large as 0.54 $\mu mol\ kg^{-1}$.

In summary, our results suggest that uptake of inorganic carbon by *P. tricornutum* is due entirely to passive diffusion of CO_2 into the cell only at very low growth rates. The results are consistent with a model that assumes that a mechanism such as active transport moves CO_2 into the cell and to the site of carboxylation in a manner that minimizes the energy required to concentrate CO_2 at the site of carboxylation. Rubisco does not have a high affinity for CO_2. Half-saturation constants for marine microalgae are on the order of 100 μM (Glover 1989; Read and Tabita 1994), the lowest reported values for marine diatoms being in the range 30–40 μM. It therefore seems unlikely that the concentration of CO_2 at the active site of Rubisco would be as low as many of the CO_2(aq) concentrations used in this study. Because typical CO_2(aq) concentrations in the ocean are 10–20 $\mu mol\ kg^{-1}$, it would not be surprising if marine microalgae had some means of concentrating CO_2 at the active site of Rubisco, and indeed several such mechanisms have been proposed (Goericke et al. 1994). Our results and model are consistent with a CO_2-concentrating mechanism, and in particular suggest that $C_i > C_e$, at least at moderate to high growth rates. Under these conditions the net diffusion of CO_2 is out of rather than into the cell.

Although our results are inconsistent with active uptake of bicarbonate from the medium, they are consistent with uptake by either active transport of CO_2 or conversion of bicarbonate to CO_2 by means of an extracellular CA followed by transport of the CO_2 into the cell via either active transport or passive diffusion. As noted by Colman and Rotatore (1995, p. 923), the contradictory evidence concerning the presence of an external CA in *P. tricornutum*, the ability

of *P. tricornutum* to concentrate inorganic carbon, and the presence of a Na^+-dependent bicarbonate uptake system "may be a consequence of the fact that different isolates of *P. tricornutum* were used . . . , and the apparently diverse characteristics reported for a single species may be a consequence of clonal differences." We agree with this assessment, but would like to mention one other consideration. Many researchers (Glover et al. 1975; Beardall et al. 1976; Rotatore et al. 1995; Colman and Rotatore 1995) have convincingly shown that *P. tricornutum* has the ability to actively take up bicarbonate. However, the experiments of Glover et al. and Beardall et al. were carried out at cell densities of 16×10^6 cells ml^{-1}, 15–20 times the cell densities in our chemostat cultures. Likewise, the experiments of Rotatore et al. and Colman and Rotatore were conducted at Chl *a* concentrations of 15–20 $\mu g\ cm^{-3}$, roughly two orders of magnitude higher than the Chl *a* concentrations in our chemostats. Under these conditions the uncatalyzed conversion of bicarbonate to CO_2(aq) would fail to meet the requirements of the algae for inorganic carbon at high growth rates, and in the absence of an external CA the algae could achieve rapid growth only by actively taking up bicarbonate. It is therefore noteworthy that in the experiments of Rotatore et al. (1995, p. 913) that "The measured CO_2 uptake rates . . . accounted for 50% of the total DIC uptake at HCO_3^-–CO_2 equilibrium." They concluded that "The cells therefore appear to take up CO_2 preferentially from the medium" (p. 914). The implication would seem to be that clones of *P. tricornutum* that lack an external CA have a facultative bicarbonate uptake system that is activated when CO_2 becomes limiting. Note, however, that Chl *a* concentrations of 15–20 $\mu g\ cm^{-3}$ are virtually unprecedented in the marine environment. Chl *a* concentrations characterized as extremely high by Yoder et al. (1994) were in fact 1,000 times smaller, and the highest Chl *a* concentration in the massive *Trichodesmium* bloom discussed by Karl et al. (1992) was only 1.1 $\mu g\ cm^{-3}$. There may indeed be times and places where marine phytoplankton lacking an external CA would need to actively take up bicarbonate to achieve rapid growth, but these circumstances are undoubtedly the exception rather than the rule. Furthermore, if the results of Burns and Beardall (1987) are not misleading, many marine phytoplankton may possess an external CA, in which case "the activity of external CA may be sufficient to provide a constant supply of CO_2, and the major inorganic carbon flux across the membrane . . . may be CO_2" (Rotatore et al. 1995, p. 917).

It should be clear from this discussion that high $\delta^{13}C$ values in phytoplankton could imply either uptake of HCO_3^-, uptake of CO_2 when the μ/CO_2(aq) ratio is high, or a combination of these phenomena. In other words, determining the form of inorganic carbon used by phytoplankton requires more information than $\delta^{13}C$ values. From the research conducted to date, it seems that phytoplankton preferentially take up CO_2, but that at least some and perhaps many species have the ability to utilize bicarbonate when the supply of CO_2 is inadequate. However, simple calculations indicate that the supply of CO_2 should rarely be limiting to phytoplankton photosynthesis in the oceans, and the laboratory conditions under which bicarbonate uptake has been clearly demonstrated seem atypical of the natural marine environment. It is reasonable to conclude that with the exception of calcareous algae (Nimer et al. 1996) virtually all synthesis of organic matter by marine phytoplankton is based on CO_2 uptake and the C_3 pathway rather than by active transport of bicarbonate.

If the relationship shown in Fig. 2 is at least qualitatively characteristic of many marine phytoplankton, then estimates of ancient CO_2(aq) concentrations based on the ϵ_p of preserved organic matter will require some estimation of the growth rates associated with production of the organic matter. If much of the preserved organic matter was produced during bloom conditions, then growth rates were likely near nutrient-saturated values, but nutrient-saturated growth rates are known to be temperature dependent (Eppley 1972; Goldman and Carpenter 1974). At least some correction for temperature effects may therefore be in order.

The existence of a unique relationship between ϵ_p and μ/CO_2(aq) could obviously be used to estimate phytoplankton growth rates in the ocean from simultaneous measurements of ϵ_p and CO_2(aq), assuming that organic matter uniquely associated with phytoplankton can be isolated and its $\delta^{13}C$ measured. Laws et al. (1995) have demonstrated the application of this approach to estimate growth rates in the equatorial Pacific using the $\delta^{13}C$ of phytoplankton Chl *a*. However, there is no reason to think that the relationship between ϵ_p and μ/CO_2(aq) is the same for all phytoplankton, for reasons discussed by Francois et al. (1993) and Laws et al. (1995). How variable the relationship may be is unclear at this time. Differences in the nature of the relationship may provide important clues as to how different species or classes of phytoplankton acquire inorganic carbon, particularly at low CO_2(aq) concentrations.

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