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 ($\mu$) 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 $\mu$mol kg$^{-1}$ and growth rates from 0.5 to 1.4 d$^{-1}$. $\epsilon_a$, the biological fractionation factor associated with carbon fixation, was found to be a nonlinear function of [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 $-$ $\epsilon_a$)/(5.3 $-\epsilon_a$)] kg d$^{-1}$ $\mu$mol$^{-1}$. This equation accounts for 92% of the variance in the $\mu$/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 linearity between the $\delta^{13}C$ of plant organic matter and the ratio of 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 ($\mu$) 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$_4$ higher plants (Kelby and Raven 1985), some of their gas exchange characteristics more closely resemble those of C$_3$ 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 (Zevenhoven and Kaplan 1981; Badger and Andrews 1972; 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-
CO$_2$ uptake by P. tricornutum

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 δ$^13$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 RuDCase 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 δ$^13$C of the phytoplankton carbon. As noted by Morel and Reinfelder (1995), however, "High δ$^13$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 Beadall (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 δ$^13$C... between HCO$_3^-$ and CO$_2$. The δ$^13$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 δ$^13$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 (Mariott 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 Beadall (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 Beadall (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 Beadall (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 Beadall (1987, p. 84) concluded that "Despite apparent utilization of HCO$_3^-$ from the bulk medium, CO$_2$ is the inorganic carbon species across 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 δ$^13$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 [μgCO$_2$(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 Beadall (1987) found evidence of a CCM. A principal motivation for the work was to determine whether the simple linear relationship between ε$_{p}$ and μgCO$_2$(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
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 CO₂ in the gas used to aerate the growth chamber was controlled using mass-flow controllers to adjust the flow rates of tank CO₂ (2.06% CO₂ in air) and CO₂-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 δ¹³C 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 δ¹³C_DIC were determined using a system modified after Kroopnick (1985). The concentration of CO₂(eq) 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 CO₂(eq) was calculated using the mass balance equation

\[ \epsilon_{\text{eq}} = \epsilon_{\text{eq}a} X_a + \epsilon_{\text{eq}c} X_c \]  

where the subscripts a, b, c, and d refer to CO₂(eq), bicarbonate, carbonate, and DIC, respectively, \( \epsilon_{\text{eq}a} \) = \( \{\delta^{13}\text{C}_a - \delta^{13}\text{C}_b\} / [1 + (\delta^{13}\text{C}_b/1,000)] \) 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. \( \epsilon_{\text{eq}a} \) and \( \epsilon_{\text{eq}c} \) 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/F 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 (Wedekind et al. 1983). Isotope abundances were measured on cryogenically purified CO₂ using either a Finnigan MAT 252 or Delta-S mass spectrometer (Santrock et al. 1985).

Particulate carbon concentrations were determined by two methods: manometricically from the amount of CO₂ 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 Celsoscope 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 \( \epsilon_p \) were calculated from the equation

\[ \epsilon_p = \frac{\delta^{13}\text{C}_a - \delta^{13}\text{C}_c}{1 + (\delta^{13}\text{C}_c/1,000)} \]  

(Freeman and Hayes 1992; Goericke et al. 1994), where \( \delta^{13}\text{C}_c \) is the δ¹³C of the aqueous CO₂ and \( \delta^{13}\text{C}_p \) is the δ¹³C of the phytoplankton carbon.

There is a highly significant negative correlation between \( \epsilon_p \) and \( \mu/\text{CO}_2(\text{aq}) \), with the Spearman rank correlation coefficient being –0.923 (\( P < 0.01 \)). However, a simple linear equation treating \( \epsilon_p \) as the independent variable gives a rather poor fit to the \( \mu/\text{CO}_2(\text{aq}) \) data (\( R^2 = 0.59 \)). The goodness-

<table>
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<th>µ (d⁻¹)</th>
<th>µCO₂ (kg d⁻¹ µmol⁻¹)</th>
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<th>δ¹³C_CO₂ (‰)</th>
<th>δ¹³C_P (‰)</th>
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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 $\mu/CO_3(aq)$ values, all of which were obtained at $CO_3(aq)$ concentrations of $<4 \mu$mol kg$^{-1}$, all lie above the linear regression line fit to the data obtained at $CO_3(aq)$ concentrations of 10–35 $\mu$mol kg$^{-1}$. We conclude that there is evidence of significant nonlinearity in the relationship between $\mu/CO_3(aq)$ and $e_p$ when the range of $\mu/CO_3(aq)$ is extended by including results from $CO_3(aq)$ concentrations below $\sim 4 \mu$mol kg$^{-1}$. Hence, we must reject the hypothesis that the underlying relationship between $e_p$ and $\mu/CO_3(aq)$ is linear over the $CO_3(aq)$ concentration range 0.64–35 $\mu$mol 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 photosynthesis are negligible (Laws et al. 1995), Francois et al. (1993) showed that $e_p$ should closely follow the equation

$$e_p = e_1 + f(e_3 - e_{-1}),$$

where $e_1$, $e_{-1}$, and $e_3$ 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 $k_{-1}C_r$, where $k_{-1}$ is the permeability of the plasmalemma to CO$_2$ and $C_r$ is the concentration of CO$_2$ in the cytoplasm. Assuming that $C_r$ 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.

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

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

$$e_p = e_1 + \frac{e_3 - e_{-1}}{1 + (\mu C/k_{-1}C_r)}.$$  

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 $e_p$ is $e_1 + e_3 - e_{-1}$; $e_{-1}$ is generally assumed to equal $\sim 0.7\%$, 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 $e_1 = e_{-1}$, in which case the maximum value of $e_p$ is $e_1 + e_3$. The combined fractionation due to Rubisco and $\beta$-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 $e_p$ is $\sim 25–28\%$, 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 $\delta^{13}C_p$ in Eq. 2 would have been replaced by $\delta^{13}C_a$, and the calculated values of $e_p$ would have increased by $\sim 9\%$. A few of the $e_p$ values would have exceeded 28%, and 9 of the 12 would have exceeded 25%. The fourth highest $e_p$ value (20.6%) is associated with a CO$_3(aq)$ of 3.6 $\mu$mol kg$^{-1}$, and the three lowest $e_p$ values are all associated with CO$_2(aq)$ of $< 2.4$ $\mu$mol 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$_3(aq)$ concentrations $> \sim 3$ $\mu$mol kg$^{-1}$. It is apparent from Fig. 1 that at the lowest CO$_3(aq)$ concentrations the phytoplankton carbon was isotopically lighter than would have been predicted from extrapolation of the trend in $e_p$ values at higher CO$_3(aq)$ concentrations. This is an improbable result if the cells had switched to bicarbonate uptake at low CO$_3(aq)$ concentrations, since the bicarbonate was isotopically heavier compared to the CO$_2(aq)$ by $\sim 9\%$. 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

$$E = F_i[A_i + B_i(C_i - C_o)] + F_o[A_o + B_o(C_o - C_i)],$$

where $F_i$ is proportional to the flux of inorganic carbon due to active transport across the plasmalemma; $F_o$ is proportional to the corresponding flux from the cytoplasm to the site of carboxylation; $C_o$, $C_i$, 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 $A_i$, $A_o$, $B_i$, and $B_o$ are constants. $A_i$ is the energetic cost of transporting one unit of CO$_2$ across the plasmalemma when $C_i = C_o$, and $B_i$ is the rate of change of that energetic cost per unit change in $C_i - C_o$. $A_o$ and $B_o$ 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 ($F_o$) proportional to the concentration gradient across the plasmalemma. If $C_o$ is constant, then $F_i = F_o = k_{-1}(C_i - C_o)$. It follows that $F_i = F_o = k_{-1}(C_i - C_o)$. It is now reasonable to equate $F_o$ to the photosynthetic rate of the cell, $\mu C$. Hence, $F_i = \mu C + k_{-1}(C_i - C_o)$. Substituting $\mu C$ for $F_o$ and $\mu C + k_{-1}(C_i - C_o)$ for $F_i$ in Eq. 6, we obtain

$$E = k_{-1}(C_i - C_o)[A_i + B_i(C_i - C_o)] + \mu C[A_i + A_o + B_i(C_i - C_o) + B_o(C_o - C_i)].$$

(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

$$\frac{dE}{dC_i} = 2k_{-1}B_i(C_i - C_o) + k_{-1}A_i - \mu C(B_o - B_i).$$

(8)

Setting this derivative equal to zero and solving for $C_i$ gives

$$C_i = C_o + \frac{\mu C(B_o - B_i) - k_{-1}A_i}{2k_{-1}B_i}.$$  

(9)

Because it follows immediately from Eq. 8 that $(d^2E/dC_i^2) = 2k_{-1}B_i > 0$, 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

$$C_o = \frac{\mu C}{k_{-1}} \left( \frac{\epsilon_o - \epsilon_i}{\epsilon_i - \epsilon_o + \epsilon_o - \epsilon_o} \right).$$

(10)

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

$$C_i = C_o + \frac{\mu C}{k_{-1}} \left( \frac{\epsilon_o - \epsilon_i}{\epsilon_i - \epsilon_o - \beta} \right),$$

(11)

where $\epsilon_i' = \epsilon_o - \epsilon_i - \epsilon_i$, $C_o = (A_o/2B_i)$ and $\beta = (B_o - B_i)/2B_i$. Rearranging Eq. 11 gives

$$\frac{\mu}{C_i - C_o} = \frac{k_{-1}}{C(1 + \beta)} \left( \epsilon_i' - \epsilon_i \right),$$

(12)

where $\epsilon_i' = \epsilon_i + [\beta/(1 + \beta)] \times (\epsilon_i' - \epsilon_i)$. Because both $\epsilon_i$ and $\epsilon_i'$ are expected to be small, it is reasonable to anticipate that $\epsilon_i'$ will be comparable to $\epsilon_i$. However, because $\epsilon_i$ is thought to be much larger than $\epsilon_i$, it is possible that $\epsilon_i'$ will differ substantially from $\epsilon_i$ if $\beta$ is not close to zero. The implication of the model is that $[\mu/(C_o - C_i)] \times [(\epsilon_o - \epsilon_i')/(\epsilon_o - \epsilon_o)]$ 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 $C_o$, $\epsilon_i'$, and $\epsilon_o'$ so as to minimize the C.V. (SD divided by the absolute value of the mean) of $[\mu/(C_o - C_i)] \times [(\epsilon_o - \epsilon_i')/(\epsilon_o - \epsilon_o)]$. The results of this exercise gave $\epsilon_i' = 5.5 \pm 0.55\%o$, $\epsilon_o' = 26.8 \pm 3.3\%o$, and $C_o = 0 \pm 0.27 \mu mol kg^{-1}$. The mean value of $[\mu/(C_o - C_i)] \times [(\epsilon_o - \epsilon_i')/(\epsilon_o - \epsilon_o)]$ was 0.225 with a C.V. of 35%. The equation describing our results therefore becomes

$$\mu/C_i = 0.225 \left( \frac{26.8 - \epsilon_o}{\epsilon_o - 5.5} \right).$$

(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 $\mu/C_i$ and clearly gives a far better
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 $\mu/C_e$.

Discussion

The results of this study leave little doubt that for *P. tricornutum* the relationship between $\varepsilon_i$ and $\mu/CO_2(aq)$ is nonlinear over the $\mu/CO_2(aq)$ range 0–2.5 kg d$^{-1}$ mol$^{-1}$. If *P. tricornutum* obtained all its carbon by simple diffusion from the bulk medium, $\varepsilon_i$ should be well approximated by a linear function of $\mu/CO_2(aq)$ (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 $\varepsilon_i$ and $\mu/CO_2(aq)$ 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 $\mu/CO_2(aq)$ is expanded by including data at $CO_2(aq) < 7$ $\mu$mol kg$^{-1}$ (Fig. 1), and the equation $\mu/C_e = 0.225 \times [(26.8 - \varepsilon_i)/(\varepsilon_i - 5.5)]$ 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 $\varepsilon_i$ and $\mu/CO_2(aq)$ for $\mu/CO_2(aq) < 0.3$ kg d$^{-1}$ mol$^{-1}$ reflects this fact. While the relationship between $\varepsilon_i$ and $\mu/CO_2(aq)$ 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$^{-1}$ mol$^{-1}$, 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 $\varepsilon_p$ should be comparable to $\varepsilon_i$. Francois et al.'s model (Eq. 5) makes no assumptions about the mechanism by which inorganic carbon enters the cell. Assuming that $\varepsilon_1 - \varepsilon_{i-1} \approx 0\%$ (Kerby and Raven 1985; Berry 1989; Hayes 1993), the parameters of our model imply that $\varepsilon_i = \varepsilon_{i-1} = 26.8\%$, within the range of probable values for $\varepsilon_i$ (25–28%) suggested by Goericke et al. (1994) and similar to Hayes’ (1993) estimate of 27%. Thus our calculated value of $\varepsilon_i$ seems reasonable. If bicarbonate were the source of the inorganic carbon entering the cell, our calculated $\varepsilon_i$ values would have to be increased by ~9%, and half would exceed 28%, the approximate upper bound on $\varepsilon_i$. Second, if the nonlinear nature of the relationship in Fig. 1 were due to active uptake of bicarbonate at high $\mu/CO_2(aq)$ 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 $\mu/CO_2(aq)$. In fact, the curvature is positive. Finally, if we were underestimating $\varepsilon_i$ 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 $\varepsilon_i$ values would become negative as $\mu/CO_2(aq)$ becomes large (Hayes 1993). In fact, all of our $\varepsilon_i$ values are positive, and the equation fit to the data predicts that $\varepsilon_i$ will exceed 5.5% for all finite $\mu/CO_2(aq)$.

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, $\mu C$. 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 particular carbon and cell count results show that *P. tricornutum* has a carbon cell content of 7.6 ± 1 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 $\mu$m$^3$. Its equivalent spherical radius is therefore ~2.6 $\mu$m. Microscopic analysis, however, reveals that its surface-to-volume ratio is about 2.4 $\mu$m$^{-1}$, equivalent to that of a sphere with a radius of 1.25 $\mu$m. 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 $\mu$m and the diameter ~1.7 $\mu$m. 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 prolunate 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 $4.38 \times 10^{-9} (C_i - C_e) m^2$ d$^{-1}$ cell$^{-1}$, where $C_i$ 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 $C_i/C_e$ ratio required to support a given growth rate $\mu$ is therefore given by the equation

$$\frac{C_i}{C_e} = 1 - (7.6 \text{ pg cell}^{-1})$$

$$+ \{4.38 \times 10^{-9} m^2 d^{-1} \text{cell}^{-1}\} \frac{\mu}{C_e}$$

$$\times \{12 \times 10^9 \text{ pg m}^{-3}(\mu\text{M}^{-1})\}$$

(14)
where the units of $\mu C_i$ are $\text{d}^{-1} \mu \text{M}^{-1}$. By far the largest $\mu C_i$ in Table 1 is 2.16 kg $\mu \text{mol}^{-1} \text{d}^{-1} = 2.11 \text{ d}^{-1} \mu \text{M}^{-1}$. Inserting this value of $\mu C_i$ into the right-hand side of Eq. 14 gives $C_i/C_o = 0.695$. This is the smallest $C_i/C_o$ 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 $\mu C_i$ ratios we examined. A corollary conclusion is that $C_i/C_o$ 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 $C_i$ and $\beta$ as previously defined, Eq. 9 can be written in the form

$$C_i = C_o + \beta \frac{\mu C}{k_{-1}}.$$  \hspace{2cm} (15)

Two important caveats to this equation are that $F_i = \mu C + k_{-1}(C_o - C_i) \geq 0$ and that $C_o \geq 0$. 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 $C_i \geq C_o - (\mu C/k_{-1})$. The constraint on Eq. 15 is therefore that $C_i - C_o + \beta (\mu C/k_{-1}) \geq C_o - (\mu C/k_{-1})$. Rearranging this inequality gives the constraint equation

$$\mu \geq \frac{C_o k_{-1}}{C(1 + \beta)}.$$  \hspace{2cm} (16)

This condition is automatically satisfied if $C_o = 0$, which is the value of $C_i$ that gives the best fit to our data. However, because the SD of $C_o$ is 0.27 $\mu \text{mol kg}^{-1}$, 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 $C_o = 0$ imposes a limit on how fast the cell can obtain CO$_2$ via diffusion. The maximum rate, corresponding to $C_o = 0$, is $C_o k_{-1}/C$. Hence, it is to be expected that the cell will obtain its inorganic carbon entirely by diffusion of CO$_2$ as long as $\mu$ is less than or equal to the smaller of $C_o k_{-1}/[C(1 + \beta)]$ and $C_o k_{-1}/C$. 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. $C_i < C_o$) as long as $\mu < (C_o k_{-1}/[\beta C])$.

Assuming that $e_i'$ is small compared to $e_0'$, our least-squares estimate of $e_i'$ should be $\sim (\beta/1 + \beta) e_0'$. With $e_0' = 5.5$ and $e_0' = 26.8$, we conclude that $\beta = 0.26$. As previously noted, $C$ for P. tricornutum is $\sim 7.6$ pg cell$^{-1}$ ($=0.63 \times 10^{-6} \mu \text{mol cell}^{-1}$). Because our least-squares analysis indicates that $k_{-1}/[C(1 + \beta)] = 0.225$, we conclude that $k_{-1} = (0.63 \times 10^{-6})(1.26)(0.225) = 1.79 \times 10^{-7}$ liters d$^{-1}$. Dividing this figure by the surface area of P. tricornutum (174 $\mu \text{m}^2$) 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 $\mu \text{mol kg}^{-1}$ and hence that $C_o k_{-1}/[C(1 + \beta)]$ could be as great as 0.54 $\times$ 0.225 (=0.122 d$^{-1}$). Likewise, $C_o k_{-1}/\beta C$ could be as great as 0.54 $\times$ 0.225 $\times$ (1 + $\beta/\beta$ = 0.54 $\times$ 0.225 $\times$ (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 $\mu C_i = 0.225 \times [(26.8 - e_i)/(e_i - 5.5)]$ gives virtually the same goodness-of-fit to the data collected at $C_o > 10 \mu \text{mol kg}^{-1}$ 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 $C_i < C_o$ 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 $\mu \text{mol kg}^{-1}$.

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 $\mu$M (Glover 1989; Read and Tabita 1994), the lowest reported values for marine diatoms being in the range 30–40 $\mu$M. 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 $C_i > C_o$, 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
CO$_2$ uptake by P. tricornutum

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 µg 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 µg 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 µg 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 δ$^{13}$C values in phytoplankton could imply either uptake of HCO$_3^-$, uptake of CO$_2$ when the μCO$_2$(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 δ$^{13}$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$_c$ 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$_c$ and μ/CO$_2$(aq) could obviously be used to estimate phytoplankton growth rates in the ocean from simultaneous measurements of ε$_c$ and CO$_2$(aq), assuming that organic matter uniquely associated with phytoplankton can be isolated and its δ$^{13}$C measured. Laws et al. (1995) have demonstrated the application of this approach to estimate growth rates in the equatorial Pacific using the δ$^{13}$C of phytoplankton Chl a. However, there is no reason to think that the relationship between ε$_c$ 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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Received: 26 September 1995
Accepted: 29 January 1997