

## CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> uptake in marine diatoms acclimated to different CO<sub>2</sub> concentrations

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### Abstract

Rates of cellular uptake of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> during steady-state photosynthesis were measured in the marine diatoms *Thalassiosira weissflogii* and *Phaeodactylum tricornutum*, acclimated to CO<sub>2</sub> partial pressures of 36, 180, 360, and 1,800 ppmv. In addition, in vivo activity of extracellular (eCA) and intracellular (iCA) carbonic anhydrase was determined in relation to CO<sub>2</sub> availability. Both species responded to diminishing CO<sub>2</sub> supply with an increase in eCA and iCA activity. In *P. tricornutum*, eCA activity was close to the detection limit at higher CO<sub>2</sub> concentrations. Simultaneous uptake of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> was observed in both diatoms. At air-equilibrated CO<sub>2</sub> levels (360 ppmv), *T. weissflogii* took up CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> at approximately the same rate, whereas CO<sub>2</sub> uptake exceeded HCO<sub>3</sub><sup>-</sup> uptake by a factor of two in *P. tricornutum*. In both diatoms, CO<sub>2</sub>:HCO<sub>3</sub><sup>-</sup> uptake ratios progressively decreased with decreasing CO<sub>2</sub> concentration, whereas substrate affinities of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> uptake increased. Half-saturation concentrations were always  $\leq 5 \mu\text{M}$  CO<sub>2</sub> for CO<sub>2</sub> uptake and  $< 700 \mu\text{M}$  HCO<sub>3</sub><sup>-</sup> for HCO<sub>3</sub><sup>-</sup> uptake. Our results indicate the presence of highly efficient uptake systems for CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> in both diatoms at concentrations typically encountered in ocean surface waters and the ability to adjust uptake rates to a wide range of inorganic carbon supply.

Primary production by marine phytoplankton takes place in an environment that is characterized by high and relatively constant HCO<sub>3</sub><sup>-</sup> concentrations (~2 mM) but low and variable concentrations of molecular dissolved CO<sub>2</sub> [CO<sub>2, aq</sub>] (~5–25  $\mu\text{M}$ ). Variation in [CO<sub>2, aq</sub>] of ocean surface waters is mainly caused by intense photosynthesis during phytoplankton blooms, differences in water temperature, or mixing with deep water of different CO<sub>2</sub> content. On longer timescales, rising CO<sub>2</sub> concentrations in the upper layers of the ocean are expected in response to the present increase in atmospheric CO<sub>2</sub> partial pressure (pCO<sub>2</sub>; Houghton et al. 1996). Because these changes in [CO<sub>2, aq</sub>] are always accompanied by changes in pH, concentrations of HCO<sub>3</sub><sup>-</sup> vary much less because of concomitant shifts in the relative proportions of the inorganic carbon (C<sub>i</sub>) species.

The response of phytoplankton growth to changes in CO<sub>2</sub> supply is largely determined by the mechanism of C<sub>i</sub> uptake. Several studies indicate that both CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> in the bulk seawater are utilized by marine eukaryotic microalgae (e.g., Colman and Rotatore 1995; Rotatore et al. 1995; Korb et al.

1997; Tortell et al. 1997; Elzenga et al. 2000). Uptake of CO<sub>2</sub> may involve passive diffusion and active transport across the plasmalemma into the cell (Miller et al. 1991; Rotatore and Colman 1991, 1992; Rotatore et al. 1992, 1995; Li and Canvin 1998). Uptake of HCO<sub>3</sub><sup>-</sup> by diffusion is restricted by the low solubility of charged molecules in membrane lipids and by the inside-negative electric potential difference across the plasmalemma in marine autotrophs (Raven 1980, 1997). Consequently, HCO<sub>3</sub><sup>-</sup> utilization occurs either by direct active uptake or by extracellular catalytic conversion of HCO<sub>3</sub><sup>-</sup> to CO<sub>2</sub> in the presence of carbonic anhydrase (CA), with CO<sub>2</sub> entering the cell (Badger et al. 1998; Kaplan et al. 1998; Sültemeyer et al. 1998). In the case of direct HCO<sub>3</sub><sup>-</sup> uptake, cellular growth is expected to be more or less unaffected by external CO<sub>2</sub>. In contrast, CA-mediated utilization of HCO<sub>3</sub><sup>-</sup> accelerates CO<sub>2</sub> formation from HCO<sub>3</sub><sup>-</sup> but has no effect on CO<sub>2</sub> equilibrium concentrations at the cell surface. Therefore, the presence of extracellular CA does not prevent phytoplankton from being affected by changes in extracellular [CO<sub>2, aq</sub>].

The proportion at which CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> are taken up and the extent to which C<sub>i</sub> uptake is affected by changes in CO<sub>2</sub> supply are still poorly quantified in marine microalgae. Evidence for HCO<sub>3</sub><sup>-</sup> utilization is often provided by methods that do not differentiate between CA-catalyzed HCO<sub>3</sub><sup>-</sup> use (with subsequent CO<sub>2</sub> uptake) and direct HCO<sub>3</sub><sup>-</sup> uptake across the plasmalemma (e.g., Burns and Beardall 1987;

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Korb et al. 1997). Other studies infer direct HCO<sub>3</sub><sup>-</sup> transport from pH drift experiments and the inhibition of light-dependent C<sub>i</sub> utilization by 4'-diisothiocyanatostilbene-2,2-disulfonic acid (DIDS), which is suggested to inhibit anion exchange processes (Nimer et al. 1997). However, no quantitative estimates of C<sub>i</sub> fluxes can be derived from this approach. Separate quantification of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> uptake, on the other hand, is usually based on measurements under conditions at which photosynthetic carbon fluxes were not at steady state (e.g., Colman and Rotatore 1995; Rotatore et al. 1995).

The method proposed by Badger et al. (1994) provides a technique to overcome the above-mentioned shortcomings. This mass spectrometric procedure uses the chemical disequilibrium between CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> during light-dependent C<sub>i</sub> uptake to differentiate between CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> fluxes across the plasmalemma and to quantify these fluxes during steady-state photosynthesis. Although this method has been successfully applied to different strains of the cyanobacterium *Synechococcus* (Badger et al. 1994; Sültemeyer et al. 1995, 1998), the halotolerant chlorophyte *Dunaliella tertiolecta* (Amoroso et al. 1998), and several freshwater microalgae, including isolated chloroplasts (Badger et al. 1994; Palmqvist et al. 1994; Amoroso et al. 1998), it has not yet been applied to marine diatoms that represent one of the dominant phytoplankton taxa in the ocean.

The present study was intended to estimate cellular uptake of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> during steady-state photosynthesis in relation to external CO<sub>2</sub> supply in two marine diatoms by use of membrane inlet mass spectrometry. We used this approach to determine substrate preferences for CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> uptake as well as possible shifts in carbon source and C<sub>i</sub> uptake kinetics in cells acclimated to different [CO<sub>2,aq</sub>]. Furthermore, the potential role of carbonic anhydrases was evaluated in these cultures on the basis of in vivo estimates of both extracellular and intracellular CA by monitoring <sup>18</sup>O exchange from doubly labelled <sup>13</sup>C<sup>18</sup>O<sub>2</sub> with the same measuring system.

## Material and methods

**Cultures, growth conditions, and sampling**—*Phaeodactylum tricornutum* (Bohlin) strain CCAP 1052/1A and *Thalassiosira weissflogii* (Grunow) Fryxell & Hasle were grown at 15°C in 0.2-μm-filtered, nutrient-enriched seawater (salinity 32) from the North Sea. Nitrate, phosphate, silicate, trace metals, ethylenediaminetetra-acetic acid (EDTA), and vitamins were added at concentrations of f/2 medium (Guillard and Ryther 1962). Batch cultures were grown in 1-liter glass tubes under continuous light at an incident photon flux density (PFD) of 120 μmol photons m<sup>-2</sup> s<sup>-1</sup>. Different CO<sub>2</sub> partial pressures (pCO<sub>2</sub>) in the medium were achieved by a continuous flow of 0.2-μm-filtered air containing 36, 180, 360, or 1,800 ppmv CO<sub>2</sub>. Because the culture medium was not buffered artificially, an increase from 36 to 1,800 ppmv CO<sub>2</sub> was accompanied by a decrease in the pH from 9.1 to 7.6. Air bubbles were dispersed at the bottom of each incubation vessel through a fritted glass disk (25 mm diameter, porosity 1). Experimental cultures were acclimated to the

respective conditions for at least 48 h before harvesting. Chlorophyll *a* concentrations at the time of sampling ranged from 34 to 183 μg L<sup>-1</sup>.

For measurements of CA activity or C<sub>i</sub> uptake, 800 ml of culture were centrifuged at 3,000 × *g* and 15°C for 5 min. Subsequently, the cells were washed twice by centrifugation (3,000 × *g*, 2 min) in CO<sub>2</sub>-free f/2 medium, buffered with 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP, 50 mM [pH 8.0], CA measurements) or 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES, 50 mM [pH 8.0], C<sub>i</sub> uptake measurements). 2 × 10 μl of the resuspended pellet were used for spectrophotometric Chl *a* analysis at 652 and 665 nm after extraction in 1 ml of methanol (5 min in darkness, room temperature), followed by centrifugation (4,500 × *g*, 2 min). A subsample of 100 ml of the original culture was used for potentiometric pH measurements and cell counts. For enumeration on a Coulter multisizer, cells were preserved with Lugol's iodine.

**Determination of dissolved inorganic carbon, alkalinity, [CO<sub>2,aq</sub>], and [HCO<sub>3</sub><sup>-</sup>]**—Cell-free subsamples of culture medium, equilibrated at the four different experimental partial pressures, were taken for measurements of total dissolved inorganic carbon (DIC) and total alkalinity (tAlk). DIC was determined coulometrically in duplicate with a system similar to that described by Johnson et al. (1993). tAlk was determined in duplicate by Gran titration with an automated, temperature-controlled system at 20°C. Concentrations of CO<sub>2</sub> ([CO<sub>2,aq</sub>]) and HCO<sub>3</sub><sup>-</sup> ([HCO<sub>3</sub><sup>-</sup>]) were calculated from DIC, tAlk, temperature, salinity, and concentrations of phosphate and silicate by use of the dissociation constants of Goyet and Poisson (1989).

**Determination of CA activity**—Activity of extracellular (eCA) and intracellular (iCA) CA was determined by measuring the loss of <sup>18</sup>O from doubly labelled <sup>13</sup>C<sup>18</sup>O<sub>2</sub> to water caused by several hydration and dehydration steps of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> (Silverman 1982; Badger and Price 1989; Sültemeyer et al. 1990). Doubly labelled CO<sub>2</sub> was prepared from H<sub>2</sub><sup>18</sup>O (98 atom% enrichment) and NaH<sup>13</sup>CO<sub>3</sub> (99 atom% enrichment), both purchased from Chemotrade (Leipzig, Germany). NaH<sup>13</sup>CO<sub>3</sub> was added to H<sub>2</sub><sup>18</sup>O (1 M C<sub>i</sub> final concentration) under N<sub>2</sub> atmosphere, sealed in an airtight glass vial, and heated at 80°C for 4 to 5 h. This procedure yielded ~80% of doubly labelled CO<sub>2</sub>. The reaction sequence of <sup>18</sup>O loss from <sup>13</sup>C<sup>18</sup>O<sup>18</sup>O (*m/z* = 49) with the intermediate product <sup>13</sup>C<sup>18</sup>O<sup>16</sup>O (*m/z* = 47), and the end product <sup>13</sup>C<sup>16</sup>O<sup>16</sup>O (*m/z* = 45) was recorded in 0.5-s intervals on a quadrupole membrane inlet mass spectrometer (MSD 5970; Hewlett Packard, Waldbronn, Germany) connected to a 10-ml thermostated cuvette. Depletion of <sup>18</sup>O in doubly labelled CO<sub>2</sub> is an irreversible reaction, because H<sub>2</sub><sup>18</sup>O produced is infinitely diluted with H<sub>2</sub><sup>16</sup>O.

For determination of CA activity, relative <sup>18</sup>O enrichment was calculated from the 45, 47, and 49 signals as a function of time according to

$$\log(\text{enrichment}) = \log[49 \times 100 / (45 + 47 + 49)]. \quad (1)$$

Typical curves for *P. tricornutum* and *T. weissflogii* are shown in Fig. 1. In a first step, 10 ml of f/2 medium, buff-

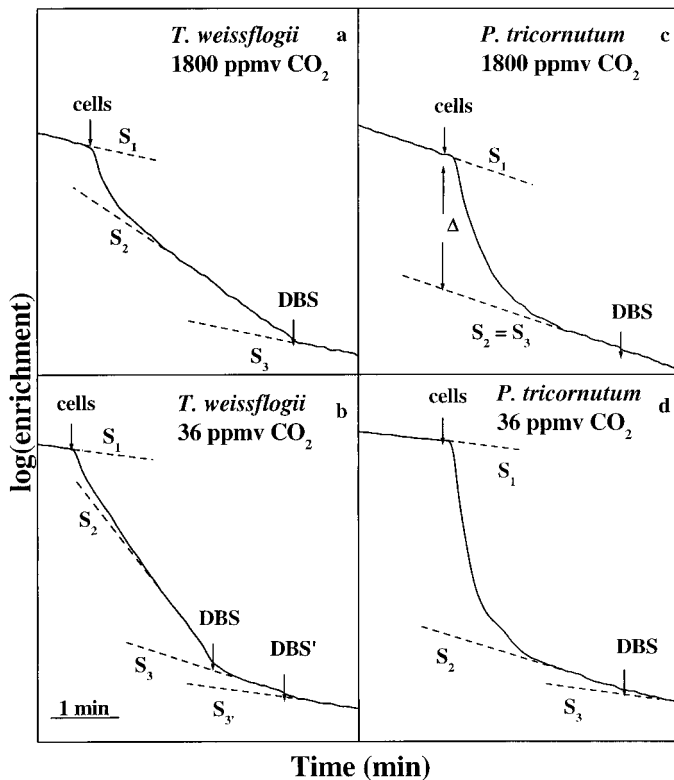


Fig. 1. In vivo measurements of CA activity based on  $^{18}\text{O}$  exchange between doubly labelled  $\text{CO}_2$  ( $^{13}\text{C}^{18}\text{O}^{18}\text{O}$ ,  $m/z = 49$ ) and water. The reaction sequence of  $^{18}\text{O}$  loss from  $m/z = 49$  to  $m/z = 45$  with the intermediate product  $m/z = 47$  was monitored by membrane inlet mass spectrometry and is reported as  $\log(\text{enrichment})$  according to Eq. 1. The initial slope ( $S_1$ ) represents  $^{18}\text{O}$  exchange during the uncatalyzed hydration/dehydration reaction between  $\text{CO}_2$  and  $\text{HCO}_3^-$ . The difference between  $S_2$  and  $S_1$  indicates  $^{18}\text{O}$  exchange in the presence of eCA. eCA activity is calculated according to Eq. 2.  $S_3$  and  $S_3'$  represent  $^{18}\text{O}$  exchange upon the addition of  $50 \mu\text{M}$  or  $100 \mu\text{M}$  DBS (an inhibitor of eCA), respectively. The drop ( $\Delta$ ) in  $\log(\text{enrichment})$  when  $S_2$  is extrapolated to the time of cell addition is taken as a measure of intracellular CA activity. (a, b) Data are shown for *T. weissflogii* and (c, d) *P. tricorutum*, (a, c) acclimated to 1,800 ppmv and (b, d) 36 ppmv in nonbuffered culture medium.

ered with 50 mM BTP (pH 8.0) was injected into the cuvette and closed with a stopper for temperature equilibration ( $15^\circ\text{C}$ ).  $10 \mu\text{l}$  of  $\text{NaH}^{13}\text{C}^{18}\text{O}_3$  solution were added to achieve 1 mM final concentration. After chemical equilibrium was reached (initial slope,  $S_1$ ),  $50\text{--}100 \mu\text{l}$  of washed cells (resuspended pellet) were added to a final Chl *a* concentration of  $1\text{--}3 \mu\text{g ml}^{-1}$ . To avoid interference of  $\text{CO}_2$  measurements with light-dependent uptake of inorganic carbon by the cells, all measurements were performed in the dark. Extracellular CA activity was estimated from the enhanced rate of  $^{18}\text{O}$  exchange, which is represented by slope  $S_2$  in Fig. 1. Intracellular CA activity was estimated from the rapid decline in  $\log(\text{enrichment})$  upon injection of cells after correction for  $S_2$  (Palmqvist et al. 1994).

Dextran-bound sulfonamide (DBS; Synthelec AB, Lund, Sweden), an inhibitor of eCA that cannot penetrate the cell

membrane (Sültemeyer et al. 1990), was used in control measurements. DBS was added to the cuvette at various concentrations either prior to injection of doubly labelled  $\text{CO}_2$  or during steady-state depletion of  $^{18}\text{O}$  due to eCA activity. The resulting rates of  $\log(\text{enrichment})$  in the presence of cells (slope  $S_3$ ) were compared with  $S_2$  in the absence of DBS to demonstrate that eCA was responsible for accelerated  $^{18}\text{O}$  depletion. Furthermore, DBS concentrations were determined at which complete inhibition of eCA activity was achieved. This inhibition is a prerequisite for measurements of  $\text{CO}_2$  uptake and  $\text{HCO}_3^-$  transport, which rely on the relatively slow spontaneous interconversion between  $\text{CO}_2$  and  $\text{HCO}_3^-$ .

*Determination of  $\text{CO}_2$  uptake,  $\text{HCO}_3^-$  uptake, and net photosynthesis*—Inorganic carbon fluxes were determined during steady-state photosynthesis by use of membrane inlet mass spectrometry. This method is described in detail by Badger et al. (1994) and has been applied in several studies since then (e.g., Palmqvist et al. 1994; Tchernov et al. 1997; Amoroso et al. 1998; Sültemeyer et al. 1998). It is based on simultaneous measurements of  $\text{O}_2$  and  $\text{CO}_2$  during consecutive light (4 min) and dark (3 min) intervals.

To estimate  $\text{CO}_2$  and  $\text{HCO}_3^-$  uptake, the same membrane inlet system was used as in CA measurements. All measurements were performed at a constant pH of 8.0. Washed cells were injected into 10 ml of  $\text{CO}_2$ -free f/2 medium, buffered with 50 mM HEPES (pH 8.0,  $15^\circ\text{C}$ ) within 10 min after centrifugation, and the cuvette was closed immediately. Chl *a* concentrations in the cuvette were  $1\text{--}3 \mu\text{g ml}^{-1}$ . DBS was added prior to cell addition at concentrations of  $50\text{--}100 \mu\text{M}$  (under the assumption of 5 mol acetazolamide covalently bound to 1 mol dextran; Palmqvist et al. 1990) to assure complete inhibition of eCA activity. After 2 min of darkness and two short light periods (0.5 min each), the first light:dark cycle was initiated at  $300 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  incident PFD and a  $\text{CO}_2$  concentration of typically  $<1 \mu\text{M}$  ( $C_i < 100 \mu\text{M}$ ). Between the subsequent light:dark cycles, known amounts of  $C_i$  were added to measure uptake rates as a function of  $\text{CO}_2$  and  $\text{HCO}_3^-$  concentrations.

Rates of  $\text{O}_2$  consumption in the dark and  $\text{O}_2$  production in the light provide a direct estimate of respiration and net  $C_i$  fixation, under the assumption of a respiratory quotient of 1.0 and a photosynthetic quotient of 1.1 to convert  $\text{O}_2$  fluxes into  $C_i$  fluxes. Gross  $\text{CO}_2$  uptake was calculated from the steady-state rate of  $\text{CO}_2$  depletion at the end of the light period and the initial rate of  $\text{CO}_2$  generation immediately after the light was turned off. This calculation is based on the assumption that the rate of diffusive  $\text{CO}_2$  efflux from a cell in the light represents the rate of  $\text{CO}_2$  efflux during the first seconds of the dark phase.

$\text{HCO}_3^-$  uptake was calculated from net  $C_i$  fixation, corrected for measured steady-state depletion of  $\text{CO}_2$  at the end of the light period and for  $\text{CO}_2/\text{HCO}_3^-$  interconversion in the medium. This calculation refers to net  $\text{HCO}_3^-$  uptake. As will be discussed below, gross  $\text{HCO}_3^-$  uptake equals net  $\text{HCO}_3^-$  only if diffusive efflux of  $\text{HCO}_3^-$  is negligible. In calculations, the required pseudo first-order rate constant  $k_2$  (formation of  $\text{CO}_2$  from  $\text{HCO}_3^-$ ) was experimentally determined from the initial slope of  $\text{CO}_2$  evolution from  $\text{HCO}_3^-$

after injection of known amounts of HCO<sub>3</sub><sup>-</sup> into CO<sub>2</sub>-free buffered medium. Several independent measurements yielded a mean value ( $\pm 1$  SD,  $n = 10$ ) of  $k_2 = 8.9 (\pm 1.1) \times 10^{-3} \text{ min}^{-1}$ . The  $k_1$  rate constant (formation of HCO<sub>3</sub><sup>-</sup> from CO<sub>2</sub>) was calculated from the product of  $k_2$  and the ratio of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> concentrations, which was measured daily in the freshly prepared assay medium. For further details regarding the method and calculations, we refer to Badger et al. (1994).

**Effect of eCA on net photosynthesis**—To investigate the potential effect of eCA on net photosynthesis, rates of photosynthetic O<sub>2</sub> evolution in the light were monitored in *P. tricornutum* and *T. weissflogii* in the presence and absence of eCA activity. Cultures acclimated to 36 or 180 ppmv CO<sub>2</sub> were used in these experiments. Changes in the O<sub>2</sub> concentration of the assay medium (10 ml, buffered with 50 mM HEPES [pH 8.0]) were monitored with the mass spectrometer by use of the same experimental protocol as for C<sub>i</sub> uptake measurements. CO<sub>2</sub> concentrations in the medium were 1 and 13  $\mu\text{M}$ . During steady-state photosynthesis in the light, DBS (100  $\mu\text{M}$  final concentration) was injected into the closed cuvette to inhibit eCA activity. In additional tests, 60  $\mu\text{g}$  of commercially available CA from bovine erythrocytes (Sigma Chemical Co., Munich, Germany; 4090 W-A units mg<sup>-1</sup> protein) was added during measurements of O<sub>2</sub> evolution in *P. tricornutum* in the absence of CA inhibitor.

## Results

**CA activity**—As indicated in Fig. 1, CA assays yielded different curves depending on species and culture conditions. After the addition of cells, a constant rate of decrease in log(enrichment) was reached within 3–6 min. The difference between  $S_2$  and  $S_1$  was caused by eCA activity, as indicated by the addition of the eCA inhibitor DBS ( $S_3$ ). Full inhibition of eCA was achieved upon the addition of DBS to 50  $\mu\text{M}$  (Fig. 1a,d) or 100  $\mu\text{M}$  (Fig. 1b) final concentration, depending on the level of activity expressed by the cells.

As a measure of eCA activity, we defined one unit as 100% stimulation of the noncatalyzed <sup>18</sup>O depletion from <sup>13</sup>C<sup>18</sup>O<sub>2</sub> according to the method of Badger and Price (1989):

$$U = (S_2 - S_1)/S_1 \quad (2)$$

For a direct comparison of eCA activity, Chl *a*-specific activities are shown in Fig. 2a.

Activity of eCA in *P. tricornutum* was close to the detection limit ( $S_1 \approx S_2 = S_3$ ; Figs. 1c and 2a), with slightly higher values only at the lowest CO<sub>2</sub> level (Figs. 1d and 2a). In *T. weissflogii*, eCA activity was significantly higher in all treatments (Figs. 1a,b and 2a). In cells acclimated to 36 ppmv CO<sub>2</sub>, eCA activity was highest, but it decreased progressively with increasing pCO<sub>2</sub>. Over the experimental CO<sub>2</sub> range, eCA activity varied by a factor of  $\sim 30$  in this species (Fig. 2a).

In all CA measurements, injection of cells into the cuvette was followed by an immediate drop in log(enrichment) (Fig. 1). This drop is caused by diffusive entry of <sup>13</sup>C<sup>18</sup>O<sub>2</sub> into the cells, in which doubly labelled CO<sub>2</sub> is initially absent. In the presence of iCA, <sup>18</sup>O loss from CO<sub>2</sub> of  $m/z = 49$  is

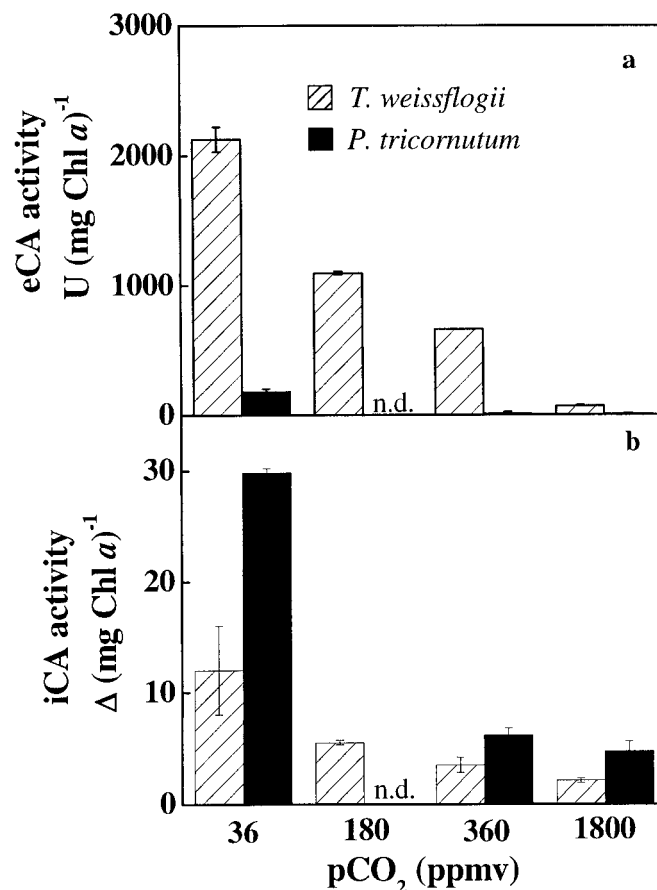


Fig. 2. Chl *a*-specific activities of (a) eCA and (b) iCA in *T. weissflogii* and *P. tricornutum* as a function of pCO<sub>2</sub> in the culture medium, to which the cells have been acclimated. n.d. = not determined.

catalyzed inside the cell, followed by diffusion of the newly generated CO<sub>2</sub> compounds ( $m/z = 47$  and  $45$ ) back to the medium (Silverman 1982). In control experiments with cells from both species acclimated to 350 ppmv CO<sub>2</sub>, the initial decline in log(enrichment) was almost 50% inhibited by 10  $\mu\text{M}$  of the membrane permeable CA-inhibitor ethoxzolamide, whereas 200  $\mu\text{M}$  were sufficient for almost complete suppression (data not shown). This indicates that internally localized CA(s) is (are) involved in the rapid exchange of doubly labeled CO<sub>2</sub> in both *P. tricornutum* and *T. weissflogii*. Both influx of  $m/z 49$  and efflux of  $m/z = 47$  and  $45$  add up to the initially observed rapid loss of <sup>18</sup>O from the medium in the presence of cells. Subsequently, iCA-mediated <sup>18</sup>O depletion from CO<sub>2</sub> in the medium solely depends on the balance between extracellular dehydration of <sup>18</sup>O-containing HCO<sub>3</sub><sup>-</sup> and the rate of CO<sub>2</sub> diffusion into the cell (Tu et al. 1986). In the absence of eCA activity, the resulting slope in our experiments approximated  $S_1$  (Fig. 1).

As a measure of iCA activity, we followed the terminology of Palmqvist et al. (1994), who defined  $\Delta$  as the drop in log(enrichment) when  $S_2$  is extrapolated to the time of cell addition (Fig. 1). Because  $\Delta$  is only determined by (1) the rate of diffusive CO<sub>2</sub> transport across cell membranes, (2)

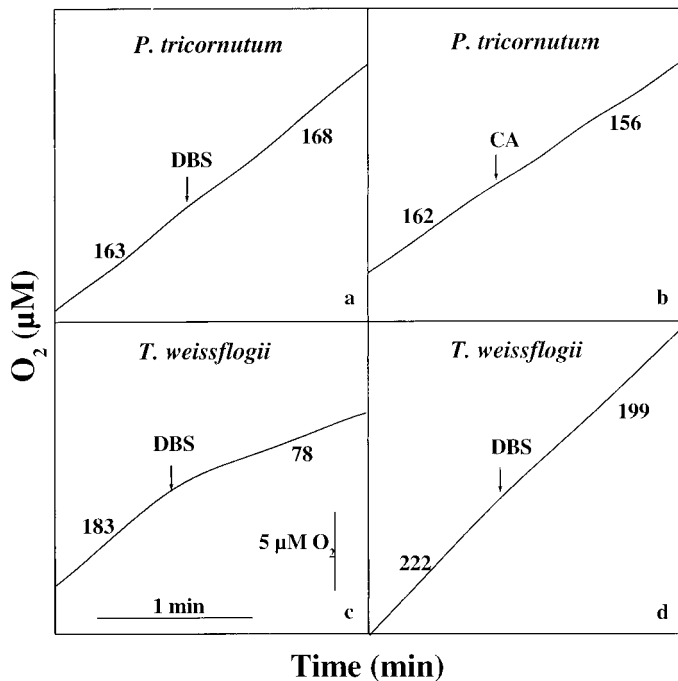


Fig. 3. Effect of the addition of the eCA-inhibitor DBS or commercially available CA on photosynthetic O<sub>2</sub> evolution in (a, b) *P. tricornutum* acclimated to 36 ppmv CO<sub>2</sub> and (c, d) *T. weissflogii* acclimated to 180 ppmv CO<sub>2</sub>. The values close to the slopes indicate Chl *a*-specific rates [ $\mu\text{mol} (\text{mg}^{-1} \text{Chl } a)^{-1} \text{h}^{-1}$ ] before and after addition of DBS/CA. Shown are typical experiments out of four to five independent replicates.

intracellular <sup>18</sup>O depletion catalyzed by iCA, and (3) the rate of extracellular <sup>18</sup>O depletion (spontaneous and eCA-catalyzed), correction for S<sub>2</sub> yields an estimate of iCA activity, expressed in arbitrary units. In contrast to measurements of eCA activity, determination of iCA activity is affected by the diffusive properties across cell membranes and the boundary layer surrounding the cells. Therefore, cell-specific characteristics such as intracellular pH, intracellular CO<sub>2</sub> concentrations, or cell size and shape potentially affect estimates of iCA activity, which prevents an interspecific comparison of enzymatic rates determined by this method. On the other hand, differences in these properties are less significant if one compares differently acclimated cells within each species.

Under the assumption that properties affecting CO<sub>2</sub> diffusion are similar for cells of a given species, Chl *a*-specific iCA estimates are directly comparable with each other. In analogy to eCA measurements, activity of iCA also depended on pCO<sub>2</sub> in the cultures. In both species we observed a significant decline in iCA activity with increasing pCO<sub>2</sub>, reflected in an approximately sixfold change over the experimental CO<sub>2</sub> range (Fig. 2b). For a direct comparison of iCA activity between differently acclimated cells of each species, Chl *a*-specific activities are shown in Fig. 2b.

Inhibition of eCA activity by DBS had no effect on net photosynthesis in *P. tricornutum* grown at 36 ppmv CO<sub>2</sub> even at [CO<sub>2,aq</sub>] = 1 μM in the assay medium (Fig. 3a),

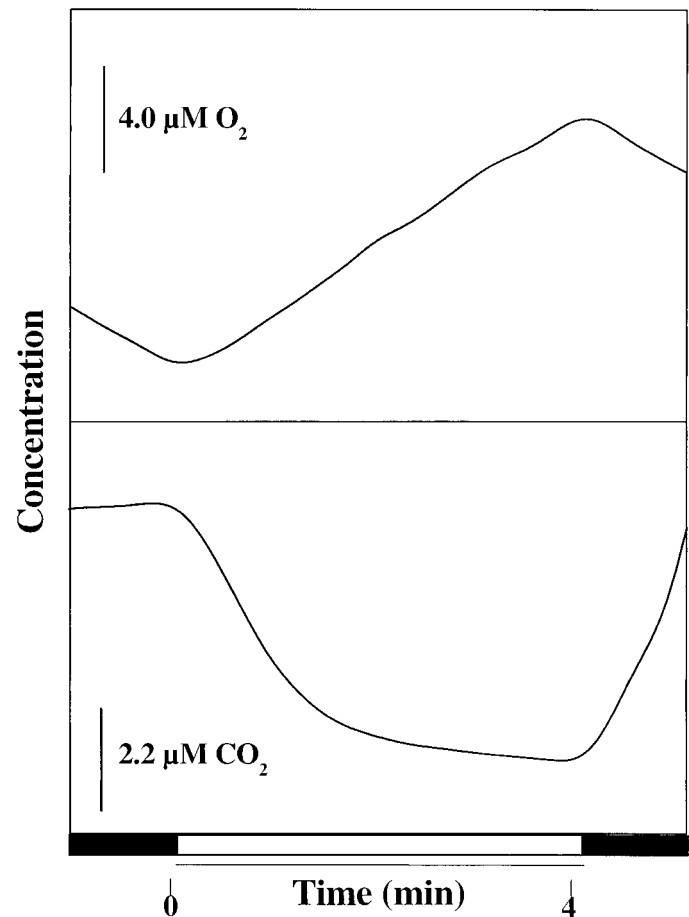


Fig. 4. Continuous measurements of O<sub>2</sub> and CO<sub>2</sub> concentrations used to estimate CO<sub>2</sub> uptake, HCO<sub>3</sub><sup>-</sup> uptake, and photosynthetic O<sub>2</sub> evolution. Data shown in this figure were obtained with *T. weissflogii*, acclimated to 1,800 ppmv CO<sub>2</sub>. The white bar at the bottom indicates a 4-min light period.

which represents the CO<sub>2</sub> concentration to which the culture was acclimated. Likewise, addition of CA had no effect on the photosynthetic rate (Fig. 3b). In *T. weissflogii* acclimated to 180 ppmv CO<sub>2</sub>, inhibition of eCA caused a decline in net photosynthesis to less than half of the original value at [CO<sub>2,aq</sub>] = 1 μM (Fig. 3c). When incubated at 13 μM of CO<sub>2</sub>, the addition of DBS still affected photosynthesis, but the rate decreased by only ~10% (Fig. 3d).

*CO<sub>2</sub> uptake, HCO<sub>3</sub><sup>-</sup> uptake, and net photosynthesis*—As indicated by a typical time course of changes in O<sub>2</sub> and CO<sub>2</sub> concentrations (Fig. 4), the transition from dark to light was accompanied by an immediate onset of photosynthetic O<sub>2</sub> production and CO<sub>2</sub> consumption. After ~3 min, the rate of decrease in CO<sub>2</sub> concentration in the cuvette reached steady state. When the light was turned off, photosynthetic O<sub>2</sub> production ceased immediately and was replaced by respiratory O<sub>2</sub> consumption. The initial CO<sub>2</sub> efflux in the dark caused the CO<sub>2</sub> concentration to increase rapidly.

Simultaneous uptake of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> during steady-state photosynthesis was observed in both diatoms (Fig. 5,

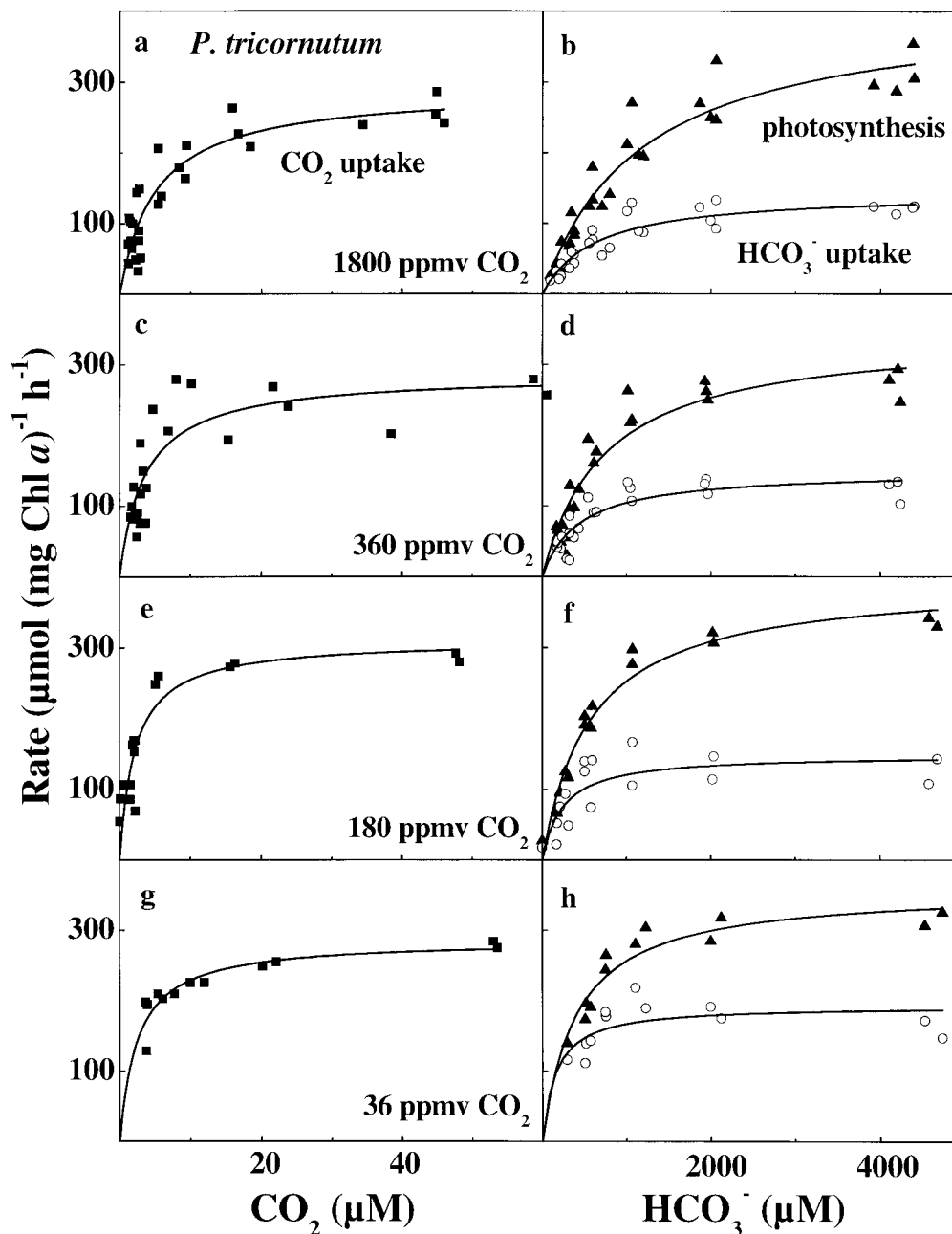


Fig. 5. *P. tricornutum*. Chl *a*-specific rates of gross CO<sub>2</sub> uptake (squares), HCO<sub>3</sub><sup>-</sup> uptake (circles), and net photosynthesis (triangles) as a function of CO<sub>2</sub> or HCO<sub>3</sub><sup>-</sup> concentration in the assay medium. Prior to the measurements, cultures were acclimated to (a, b) 1,800 ppmv; (c, d) 360 ppmv; (e, f) 180 ppmv; or (g, h) 36 ppmv of CO<sub>2</sub> for at least 48 h. Curves were obtained from a Michaelis-Menten fit.

6). However, species-specific substrate preferences for inorganic carbon uptake and differences in the effect of culture pCO<sub>2</sub> on uptake kinetics existed. Maximum rates ( $V_{\max}$ ) and half-saturation concentrations ( $K_{1/2}$ ) of inorganic carbon uptake and photosynthetic O<sub>2</sub> evolution were calculated from a Michaelis-Menten fit to the combined data of several independent measurements (Fig. 7).

In *P. tricornutum* maximum rates of gross CO<sub>2</sub> uptake,

HCO<sub>3</sub><sup>-</sup> uptake, and net O<sub>2</sub> evolution were largely unaffected by acclimation to different pCO<sub>2</sub> (Figs. 5 and 7b). In general, rates of CO<sub>2</sub> uptake were approximately twice the rates of HCO<sub>3</sub><sup>-</sup> uptake, which indicates that CO<sub>2</sub> was the preferred substrate for inorganic carbon uptake in this species. In *T. weissflogii*, maximum rates of HCO<sub>3</sub><sup>-</sup> uptake and net O<sub>2</sub> evolution also remained largely unaffected by acclimation to a wide range of pCO<sub>2</sub> (Figs. 5 and 7a). A significant decrease

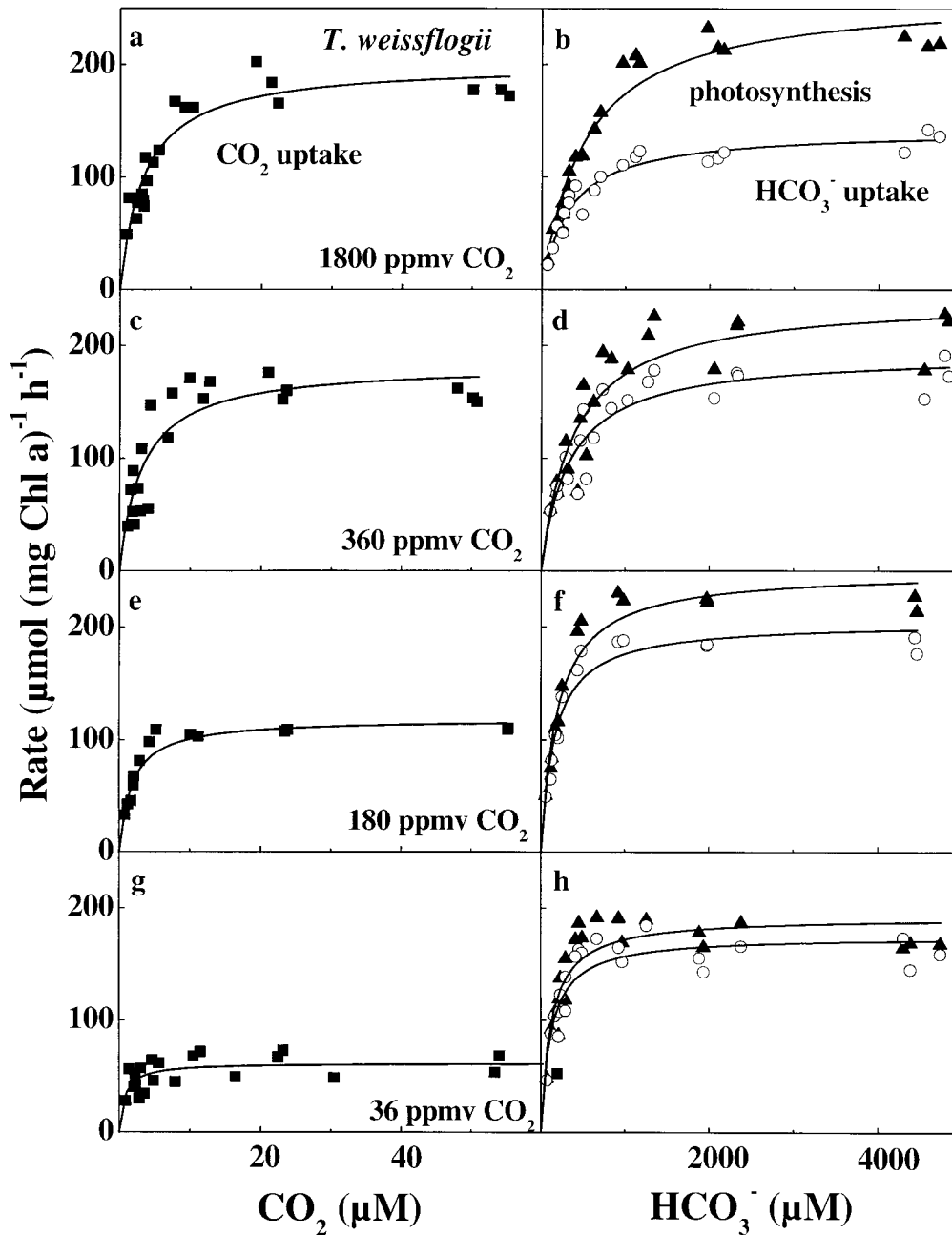


Fig. 6. *T. weissflogii*. Chl *a*-specific rates of gross CO<sub>2</sub> uptake (squares), HCO<sub>3</sub><sup>-</sup> uptake (circles), and net photosynthesis (triangles) as a function of CO<sub>2</sub> or HCO<sub>3</sub><sup>-</sup> concentration in the assay medium. Prior to the measurements, cultures were acclimated to (a, b) 1,800 ppmv; (c, d) 360 ppmv; (e, f) 180 ppmv; or (g, h) 36 ppmv of CO<sub>2</sub> for at least 48 h. Curves were obtained from a Michaelis-Menten fit.

in  $V_{\max}$  was only observed for HCO<sub>3</sub><sup>-</sup> uptake at the highest pCO<sub>2</sub> and for photosynthetic O<sub>2</sub> evolution at the lowest pCO<sub>2</sub>. Unlike *P. tricornutum*, however,  $V_{\max}$  of gross CO<sub>2</sub> uptake continuously decreased with decreasing pCO<sub>2</sub>. This indicates that CO<sub>2</sub> was preferentially taken up by cells acclimated to 1,800 ppmv CO<sub>2</sub>, whereas HCO<sub>3</sub><sup>-</sup> uptake dominated in cells grown at 36 ppmv CO<sub>2</sub>.

An increase in substrate affinities of carbon uptake upon

acclimation to decreasing CO<sub>2</sub> supply was observed in both diatoms, as was indicated by a decrease in  $K_{1/2}(\text{CO}_2)$  of CO<sub>2</sub> uptake and in  $K_{1/2}(\text{HCO}_3^-)$  of HCO<sub>3</sub><sup>-</sup> uptake with decreasing pCO<sub>2</sub> in the cultures (Fig. 7c,d). Half-saturation of CO<sub>2</sub> uptake generally occurred at  $[\text{CO}_{2,\text{aq}}] \leq 5 \mu\text{M}$  in both species. Half-saturation concentrations of HCO<sub>3</sub><sup>-</sup> uptake were slightly lower in *T. weissflogii* than in *P. tricornutum* but were always <700 μM.

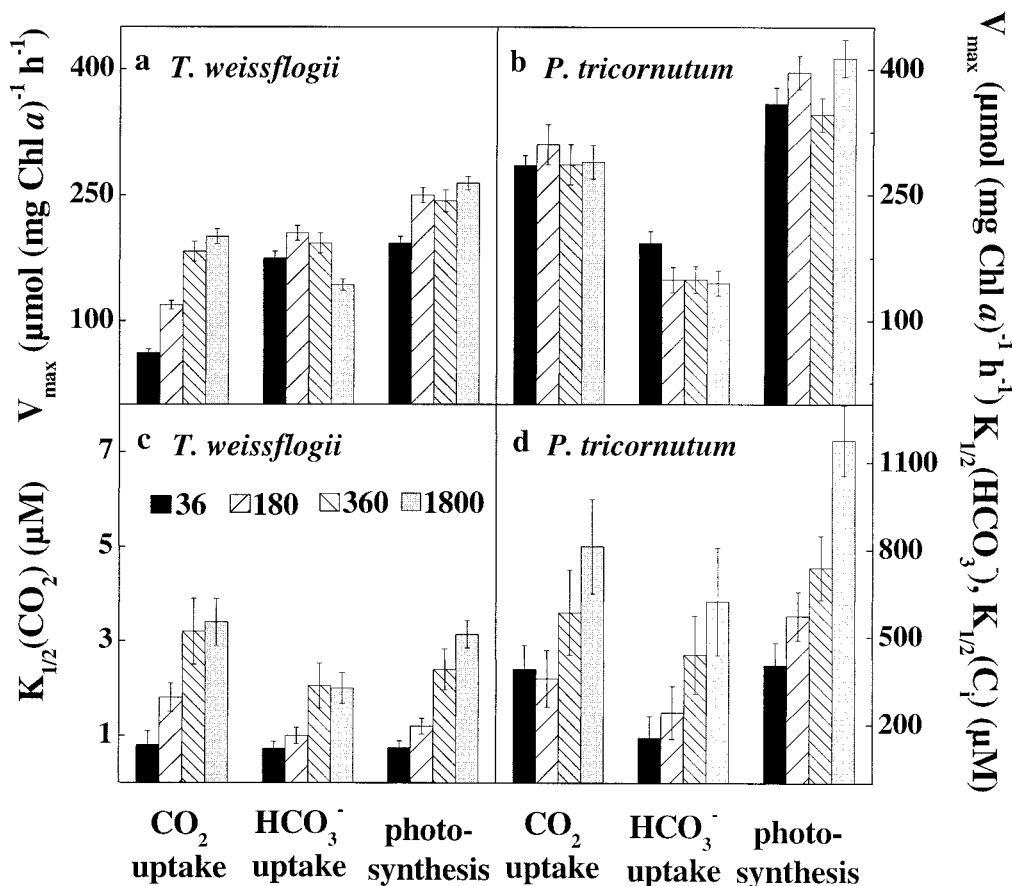


Fig. 7.  $V_{\max}$  and  $K_{1/2}$  of gross CO<sub>2</sub> uptake, HCO<sub>3</sub><sup>-</sup> uptake, and photosynthetic O<sub>2</sub> evolution for (b, d) *P. tricornutum* and (a, c) *T. weissflogii*. Kinetic parameters were calculated from a Michaelis-Menten fit to the combined data of several independent measurements. Error bars indicate  $\pm 1$  SD.

To evaluate the effect of a further decline in inorganic carbon availability on uptake kinetics, cultures of *P. tricornutum*, aerated with 36 ppmv CO<sub>2</sub> were harvested at a higher Chl *a* concentration (250  $\mu\text{g l}^{-1}$ ). In these cultures, the rate of CO<sub>2</sub> uptake exceeded the rate of CO<sub>2</sub> supply, so that CO<sub>2</sub>

Table 1. *P. tricornutum*.  $V_{\max}$ ,  $K_{1/2}(\text{CO}_2)$ , and  $K_{1/2}(\text{HCO}_3^-)$  ( $\pm 1$  SD) of CO<sub>2</sub> uptake and HCO<sub>3</sub><sup>-</sup> uptake. All cultures were aerated with 36 ppmv CO<sub>2</sub>. Cultures growing at equilibrium with CO<sub>2</sub> in the air stream are compared with cultures (both nonbuffered and buffered at pH 8.0), harvested at higher Chl *a* concentrations, at which the rate of photosynthetic CO<sub>2</sub> consumption exceeded the rate of CO<sub>2</sub> supply.

	CO <sub>2</sub> supply =	CO <sub>2</sub> supply < CO <sub>2</sub>	
	consumption,	Nonbuffered	Buffered
	nonbuffered		at pH 8.0
CO <sub>2</sub> uptake			
$V_{\max}$	285 (12)	282 (16)	256 (12)
$K_{1/2}(\text{CO}_2)$	2.4 (0.5)	1.7 (0.4)	1.5 (0.3)
HCO <sub>3</sub> <sup>-</sup> uptake			
$V_{\max}$	193 (14)	201 (10)	185 (9)
$K_{1/2}(\text{HCO}_3^-)$	155 (74)	12 (8)	15 (15)

concentrations were no longer at equilibrium with the air stream. Consequently, [CO<sub>2,aq</sub>] dropped to 0.01  $\mu\text{M}$ , HCO<sub>3</sub><sup>-</sup> decreased to 117  $\mu\text{M}$ , and pH increased to 10.1. Compared with experiments at 36 ppmv CO<sub>2</sub> under equilibrium conditions,  $V_{\max}$  of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> uptake as well as  $K_{1/2}(\text{CO}_2)$  of CO<sub>2</sub> uptake were largely unaffected by the further C<sub>i</sub> depletion (Table 1). On the other hand, we observed a decline in  $K_{1/2}(\text{HCO}_3^-)$  of HCO<sub>3</sub><sup>-</sup> uptake by a factor of 10 under these conditions.

Similar kinetic constants were obtained in *P. tricornutum* cultivated at 36 ppmv CO<sub>2</sub> in the presence of buffer (20 mM HEPES) at a constant pH of 8.0 (Table 1). Equilibrium concentrations of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> were 1.4 and 116  $\mu\text{M}$  in the buffered cultures, but lower concentrations are, again, expected when cells are harvested at 250  $\mu\text{g l}^{-1}$  Chl *a*. In both buffered and nonbuffered cultures, these extremely low concentrations of inorganic carbon yielded values of  $K_{1/2}(\text{CO}_2) = 1.5\text{--}1.7 \mu\text{M}$  for CO<sub>2</sub> uptake and  $K_{1/2}(\text{HCO}_3^-) = 12\text{--}15 \mu\text{M}$  for HCO<sub>3</sub><sup>-</sup> uptake, regardless of a difference in pH by two units.

## Discussion

Results from the present study indicate that small modifications of mass-spectrometric techniques, previously estab-



lished to estimate inorganic carbon fluxes in cyanobacteria and freshwater microalgae (Badger et al. 1994; Palmqvist et al. 1994), provide the opportunity for such measurements in marine diatoms. This opens the field for the examination of organisms that constitute a large portion of marine primary production.

Several key aspects of inorganic carbon acquisition in the two diatoms tested here closely resemble general patterns previously observed in the freshwater chlorophytes *Chlamydomonas reinhardtii*, *Scenedesmus obliquus*, and the halotolerant *D. tertiolecta* (Badger et al. 1994; Palmqvist et al. 1994; Amoroso et al. 1998). These aspects include the capacity for simultaneous transport of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> during photosynthesis even after growth at high CO<sub>2</sub> concentrations and the increase in the apparent affinities of both transport systems in response to diminishing supply of carbon substrate. Furthermore, iCA activity and, if present, eCA activity increased in response to a decrease in CO<sub>2</sub> concentration.

In spite of similarities in carbon acquisition among different taxa, we observed a number of species-specific characteristics. Most obvious is the high contribution of CO<sub>2</sub> uptake to total C<sub>i</sub> uptake in *P. tricornutum* compared with the greater proportion of HCO<sub>3</sub><sup>-</sup> uptake in *T. weissflogii*. Furthermore, the maximum rate of CO<sub>2</sub> uptake in *P. tricornutum* remains largely unaffected by variable pCO<sub>2</sub>, whereas it decreases upon CO<sub>2</sub> depletion in *T. weissflogii*. Changes in V<sub>max</sub> may indicate a change in the number of transport components in response to substrate availability. Another important difference between the species tested is the level of eCA activity. In fact, one reason for the choice of strain CCAP 1052/1A of *P. tricornutum* was the reported lack of eCA activity (John-McKay and Colman 1997), given that the absence of eCA activity is a prerequisite for C<sub>i</sub> uptake measurements. In the absence of eCA, no inhibitor needs to be added during the assay.

Our results indicate that eCA activity of strain CCAP 1052/1A was close to the detection limit when cells were acclimated to pCO<sub>2</sub> levels of 360 ppmv or higher and remained low even in the lowest pCO<sub>2</sub> treatment. This finding is consistent with observations by John-McKay and Colman (1997) who—in a comparison of 11 strains of *P. tricornutum*—detected eCA activity in all but 3 strains of this species. Strain CCAP 1052/1A was among those three strains that lacked significant external CA activity. Because *P. tricornutum* showed a preference for CO<sub>2</sub> as the inorganic carbon source in our experiments, the low level of eCA expression in this organism is surprising. Interestingly, the addition of the eCA inhibitor DBS or addition of commercially available CA during measurements of photosynthetic rates both had no effect on net C<sub>i</sub> fixation in this strain. This indicates that this strain of *P. tricornutum* has optimized its carbon uptake systems in a way that makes it largely independent of extracellular catalytic conversion of HCO<sub>3</sub><sup>-</sup> to CO<sub>2</sub>. In contrast, eCA activity appears to be crucial in other strains of *P. tricornutum*, as indicated by a significant decrease in photosynthetic CO<sub>2</sub> fixation by strain CCAP 1052/6 upon the addition of DBS in a study by Iglesias-Rodriguez and Merrett (1997). It should be noted here that the lack of response to the addition of DBS in our experiments with

strain CCAP 1052/1A demonstrates that the inhibitor itself has no effect on photosynthetic rates.

In *T. weissflogii*, where HCO<sub>3</sub><sup>-</sup> appears to be the dominant C<sub>i</sub> species taken up by the cells, especially at low CO<sub>2</sub> concentrations, one would expect that eCA activity plays a minor role in carbon acquisition. Nevertheless, this diatom expressed relatively high eCA activity, with a 30-fold increase in response to decreasing CO<sub>2</sub> availability. That eCA activity is beneficial for photosynthetic carbon fixation of *T. weissflogii* is supported by significantly higher photosynthetic rates in the absence of eCA inhibitor. In cells adapted to 180 ppmv CO<sub>2</sub>, this effect was large at low [CO<sub>2, aq</sub>] (1 μM) but small at higher [CO<sub>2, aq</sub>] (13 μM). At 1 μM of CO<sub>2</sub>, corresponding to ~90 μM of HCO<sub>3</sub><sup>-</sup> at pH 8.0, cells acclimated to 180 ppmv CO<sub>2</sub> exhibited similar rates of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> uptake. This indicates that, under limiting C<sub>i</sub> concentrations, CO<sub>2</sub> supply is important for the cells.

Mass-spectrometric measurements of iCA activity have the advantage that highly reproducible results can be derived from living cells. For a given species, apparent activities are directly comparable after normalization for Chl *a* or cell numbers. Our in vivo estimates of iCA activity confirm the commonly accepted notion that the majority of microalgal species responds to CO<sub>2</sub> depletion with an increase in iCA activity. As in several other enzyme assays, a major drawback of this method is that the enzyme activity is in relative units, which cannot be directly converted into absolute rates (mol substrate catalyzed per unit of time). In addition, comparisons between species should be treated with caution, because measurements of internal CA activity that use the parameter Δ depend on the diffusive influx of doubly labeled CO<sub>2</sub> and thus on the diffusive properties of algal membranes. Therefore, it is reasonable to conclude that both species respond to a decrease in pCO<sub>2</sub> from 1,800 to 36 ppmv with an increase in iCA activity by a factor of six, which underlines the importance of iCA in inorganic carbon acquisition, although its function in eukaryotic microalgae is still not fully understood (Sültemeyer 1998). On the other hand, it is difficult to assess whether Δ values in *P. tricornutum* reflect higher iCA activity than in *T. weissflogii* or whether they are caused by more rapid diffusion of labelled CO<sub>2</sub> across the plasmalemma in the smaller species *P. tricornutum*.

Prior to the discussion of C<sub>i</sub> flux estimates, we would like to summarize some of the assumptions underlying our calculations and how they potentially affect the results. A critical assessment of the method we used to estimate C<sub>i</sub> fluxes is provided by Badger et al. (1994) and by Kaplan and Reinhold (1999). Critical assumptions that may affect C<sub>i</sub> flux estimates concern the quantification of CO<sub>2</sub> efflux and the possibility of HCO<sub>3</sub><sup>-</sup> efflux from the cells back to the medium. Quantification of CO<sub>2</sub> efflux assumes that the rate of diffusive CO<sub>2</sub> efflux in the light is well represented by the rate of CO<sub>2</sub> efflux during the first seconds of the subsequent dark phase. According to Badger et al. (1994), this method may underestimate actual rates of CO<sub>2</sub> efflux in eukaryotic microalgae as a result of cell compartmentation. Furthermore, we cannot exclude the possibility that an energy-driven transport of CO<sub>2</sub> out of the cell takes place in the light, leading to an underestimate of CO<sub>2</sub> efflux when it is determined from initial rates of CO<sub>2</sub> efflux during the first sec-

onds of the dark phase. In fact, massive CO<sub>2</sub> efflux originating from HCO<sub>3</sub><sup>-</sup> uptake with subsequent intracellular conversion to CO<sub>2</sub> was proposed by Tchernov et al. (1997, 1998) on the basis of experiments with the cyanobacterium *Synechococcus* and with *Nannochloropsis* (Eustigmatophyceae). Any underestimate of CO<sub>2</sub> efflux would imply an underestimate of gross CO<sub>2</sub> uptake rates presented in this study.

Evidence for HCO<sub>3</sub><sup>-</sup> efflux has, until now, only been reported for cyanobacteria (e.g., Salon et al. 1996; Kaplan and Reinhold 1999). Whether similar rates of HCO<sub>3</sub><sup>-</sup> efflux can be achieved in eukaryotic microalgae, which accumulate intracellular C<sub>i</sub> to a lesser degree and impose additional membrane barriers for HCO<sub>3</sub><sup>-</sup> efflux, remains to be tested. Interpretation of HCO<sub>3</sub><sup>-</sup> efflux is complicated by the use of inhibitors of photosynthetic carbon fixation, such as iodoacetamid, which are applied during these measurements. This approach has the advantage that HCO<sub>3</sub><sup>-</sup> efflux can be measured while light energy is available for active transport processes. Because of the low permeability of biological membranes for HCO<sub>3</sub><sup>-</sup>, any HCO<sub>3</sub><sup>-</sup> loss from a cell is likely to involve transport processes other than passive diffusion. On the other hand, evidence for HCO<sub>3</sub><sup>-</sup> efflux during inhibition of C<sub>i</sub> fixation still leaves the question whether this loss of inorganic carbon from the cell would also occur without impeding photosynthetic carbon fixation. No indication of HCO<sub>3</sub><sup>-</sup> efflux was detected in *P. tricornutum* in the light (300 μmol photons m<sup>-2</sup> s<sup>-1</sup>) when treated with 5 mM iodoacetamid (Sültemeyer unpubl. data).

If significant HCO<sub>3</sub><sup>-</sup> efflux occurred in our assays, our estimates of HCO<sub>3</sub><sup>-</sup> uptake would represent net uptake of HCO<sub>3</sub><sup>-</sup>. In other words, the method would still be capable of demonstrating the ability of direct HCO<sub>3</sub><sup>-</sup> uptake accompanied by CO<sub>2</sub> uptake as an important additional carbon source in photosynthesis. However,  $K_{1/2}(\text{HCO}_3^-)$  estimates obtained with this approach (Fig. 7) would be erroneous if large HCO<sub>3</sub><sup>-</sup> efflux occurred. As long as we are unable to provide accurate measurements of HCO<sub>3</sub><sup>-</sup> efflux during photosynthesis in the absence of inhibitors, the assumptions made in our calculations should be kept in mind when interpreting C<sub>i</sub> uptake kinetics.

Our observation of simultaneous CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> uptake in marine diatoms is consistent with previous investigations (e.g., Colman and Rotatore 1995; Rotatore et al. 1995). In addition, results from the present study are the first individual estimates of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> uptake rates during steady-state photosynthesis in marine diatoms that can be used to determine uptake kinetics for both uptake systems as a function of their respective substrates. Three main conclusions can be drawn from the high substrate affinities observed in the present study: (1) Even in cells acclimated to the highest pCO<sub>2</sub> level, half-saturation of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> uptake was achieved at concentrations of 3.4–5.0 μM CO<sub>2</sub> and 325–623 μM HCO<sub>3</sub><sup>-</sup>. Compared with typical [CO<sub>2, aq</sub>] of >10 μM and [HCO<sub>3</sub><sup>-</sup>] of ~2 mM in ocean surface waters, these results provide evidence for highly efficient CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> uptake in both diatoms. (2) As indicated by a continuous decrease in  $K_{1/2}(\text{CO}_2)$  of CO<sub>2</sub> uptake and  $K_{1/2}(\text{HCO}_3^-)$  of HCO<sub>3</sub><sup>-</sup> uptake, both organisms were able to respond to diminishing carbon supply with a further increase in substrate affinity of their transport systems. (3) Similar to CO<sub>2</sub> uptake kinetics,

$K_{1/2}(\text{CO}_2)$  values of photosynthesis range from 1.3 to 5.8 μM CO<sub>2</sub>, depending on species and culture conditions. In contrast, the few data available for kinetic properties of the CO<sub>2</sub>-fixing enzyme Rubisco in diatoms are approximately one order of magnitude higher. According to Badger et al. (1998), half-saturation of the Rubisco carboxylase reaction is achieved at 31–36 μM in two strains of *Cylindrotheca* and at 41 μM in *P. tricornutum*. Higher CO<sub>2</sub> affinity of photosynthesis than of the Rubisco reaction provides evidence for the presence of a carbon-concentrating mechanism (CCM) that is maintained by active C<sub>i</sub> transport.

Culture conditions in our experiments were chosen to simulate natural variations in surface ocean carbonate chemistry as closely as possible. Because any change in CO<sub>2</sub> concentration under natural conditions is accompanied by a change in pH, we purposely avoided adding buffer to the culture medium. Consequently, an increase from 36 to 1,800 ppmv CO<sub>2</sub> was accompanied by a decrease in pH from 9.1 to 7.6 and a decrease in the [HCO<sub>3</sub><sup>-</sup>]:[CO<sub>2, aq</sub>] ratio from 767 to 32. As a result of the variable [HCO<sub>3</sub><sup>-</sup>]:[CO<sub>2, aq</sub>] ratio, a 50-fold increase in [CO<sub>2, aq</sub>] led to an increase in [HCO<sub>3</sub><sup>-</sup>] by only a factor of two. In this respect, buffered and nonbuffered seawater media differ considerably. Whereas both media aerated at different pCO<sub>2</sub> experience the same variation in [CO<sub>2, aq</sub>] (1.4–70.5 μM in this study), the variation in [HCO<sub>3</sub><sup>-</sup>] is a lot less in buffered than in nonbuffered media (1.1–2.3 mM in this study, compared with 0.1–6.1 mM when buffered at pH 8.0).

The present study was not intended to evaluate the effect of buffer on C<sub>i</sub> uptake kinetics. However, one experiment with *P. tricornutum*, in which extremely low CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> concentrations were achieved by photosynthetic C<sub>i</sub> consumption (exceeding C<sub>i</sub> supply), was performed with both buffered and nonbuffered culture medium. Results indicate that maximum substrate affinity of both CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> transport systems can be induced regardless of a difference in pH by two units (Table 1). In these experiments,  $K_{1/2}(\text{CO}_2)$  of CO<sub>2</sub> uptake was only slightly lower than in nonbuffered cultures growing at equilibrium with 36 ppmv CO<sub>2</sub> in the air stream. In contrast,  $K_{1/2}(\text{HCO}_3^-)$  of HCO<sub>3</sub><sup>-</sup> uptake decreased drastically from 155 μM (at equilibrium with 36 ppmv CO<sub>2</sub>, [HCO<sub>3</sub><sup>-</sup>] = 1,074 μM) to 12–15 μM (excess C<sub>i</sub> consumption, [HCO<sub>3</sub><sup>-</sup>] < 117 μM HCO<sub>3</sub><sup>-</sup>). This indicates that the high-affinity HCO<sub>3</sub><sup>-</sup> uptake system of *P. tricornutum* is not fully induced in nonbuffered cultures, even when they grow at equilibrium with 36 ppmv CO<sub>2</sub>.

Evidence from studies of cyanobacteria suggests that CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> transport are separate, independent processes (Espie et al. 1991; Omata et al. 1999) and that HCO<sub>3</sub><sup>-</sup> is involved as a primary signal to induce high-affinity HCO<sub>3</sub><sup>-</sup> transport (Sültemeyer et al. 1998). Our results are consistent with these observations, although we cannot rule out the possibility that changes in CO<sub>2</sub> supply also affect HCO<sub>3</sub><sup>-</sup> acquisition in the two diatoms of this study. If the CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> uptake systems operate independent of each other, this has important consequences for the interpretation of our results. As in any mass-spectrometric analysis, our measurements require constant pH in order to keep the rate constants of CO<sub>2</sub>-HCO<sub>3</sub><sup>-</sup> interconversion reactions invariable. Therefore, cells suspended in buffered assay medium experience,

in contrast to nonbuffered culture medium, constant  $[\text{HCO}_3^-]:[\text{CO}_{2,\text{aq}}]$  ratios during flux measurements at any  $C_i$  concentration. Although this approach is appropriate to determine changes in  $\text{CO}_2$  and  $\text{HCO}_3^-$  uptake capacities in relation to variable supply of the respective  $C_i$  species, calculated uptake kinetics are not truly representative of cells that have been acclimated in the absence of buffer.

To account for variable  $[\text{HCO}_3^-]:[\text{CO}_{2,\text{aq}}]$  ratios during acclimation, we applied measured uptake kinetics (Fig. 7) to calculate  $\text{CO}_2$  and  $\text{HCO}_3^-$  uptake rates as a function of the actual  $\text{CO}_2$  and  $\text{HCO}_3^-$  concentrations to which the diatoms were exposed in the cultures. These calculations are based on the assumption that  $\text{CO}_2$  and  $\text{HCO}_3^-$  transport systems are regulated independent of each other and that changes in pH have no significant effect on uptake kinetics. One important implication of such calculations is the effect on  $\text{CO}_2:\text{HCO}_3^-$  uptake ratios at different culture conditions. In both species, we observe a systematic decrease in the  $\text{CO}_2:\text{HCO}_3^-$  uptake ratio in response to a decrease in  $p\text{CO}_2$  to which the cultures were acclimated (Fig. 8a). The horizontal line indicates the value at which  $\text{CO}_2$  and  $\text{HCO}_3^-$  are taken up at equal proportions. It becomes evident that *P. tricornutum* prefers  $\text{CO}_2$  as a substrate at all but the lowest  $p\text{CO}_2$ . In contrast, *T. weissflogii* shifts from  $\text{CO}_2$  to  $\text{HCO}_3^-$  as preferred carbon substrate at  $p\text{CO}_2 \leq 360$  ppmv. These results demonstrate that a decline in  $\text{CO}_2$  supply is accompanied by the gradual induction of  $\text{HCO}_3^-$  uptake, whereas both species favor the uptake of  $\text{CO}_2$  if present at high concentrations.

Such a regulatory mechanism raises the questions why marine diatoms, which have the capacity for active  $\text{HCO}_3^-$  transport, do not rely on the abundant  $\text{HCO}_3^-$  pool in seawater as the only inorganic carbon source. We suggest that the coexistence of both transport systems with preference for  $\text{CO}_2$  uptake at higher  $\text{CO}_2$  concentrations is maintained for energetic reasons. Even in the case of active  $\text{CO}_2$  transport, its energetic cost is expected to be lower than that of  $\text{HCO}_3^-$  uptake, owing to the negative charge of this molecule and the inside-negative electric potential difference across the plasmalemma. Because marine phytoplankton evolved in an environment in which light supply is frequently limiting growth, the operation of a high-affinity  $\text{CO}_2$  transport system may be advantageous from an energetic point of view.

Another way to minimize the substantial energetic costs associated with  $C_i$  uptake is to avoid inorganic carbon loss from the cell. Because we measured total  $C_i$  uptake and photosynthetic carbon fixation, mass balance calculations provide an estimate of inorganic carbon loss. The underlying assumptions are that carbon leakage from inside the cells back to the medium occurs mainly by diffusion of  $\text{CO}_2$  and that the efflux of  $\text{HCO}_3^-$  is negligible. In this case, our calculations of  $C_i$  leakage (defined as the ratio of  $\text{CO}_2$  efflux to total  $C_i$  uptake) indicate that  $\sim 30\%$  of the inorganic carbon taken up is subsequently lost in both diatoms, when acclimated to  $p\text{CO}_2 \leq 360$  ppmv (Fig. 8b). In response to a decrease in  $p\text{CO}_2$  to 36 ppmv, leakage is minimized to values  $< 10\%$ . Such an increase in  $C_i$  assimilation efficiency may serve to reduce the higher energetic cost associated with an increased proportion of  $\text{HCO}_3^-$  taken up by low- $\text{CO}_2$  cells.

Our calculations of variable  $\text{CO}_2:\text{HCO}_3^-$  uptake ratios

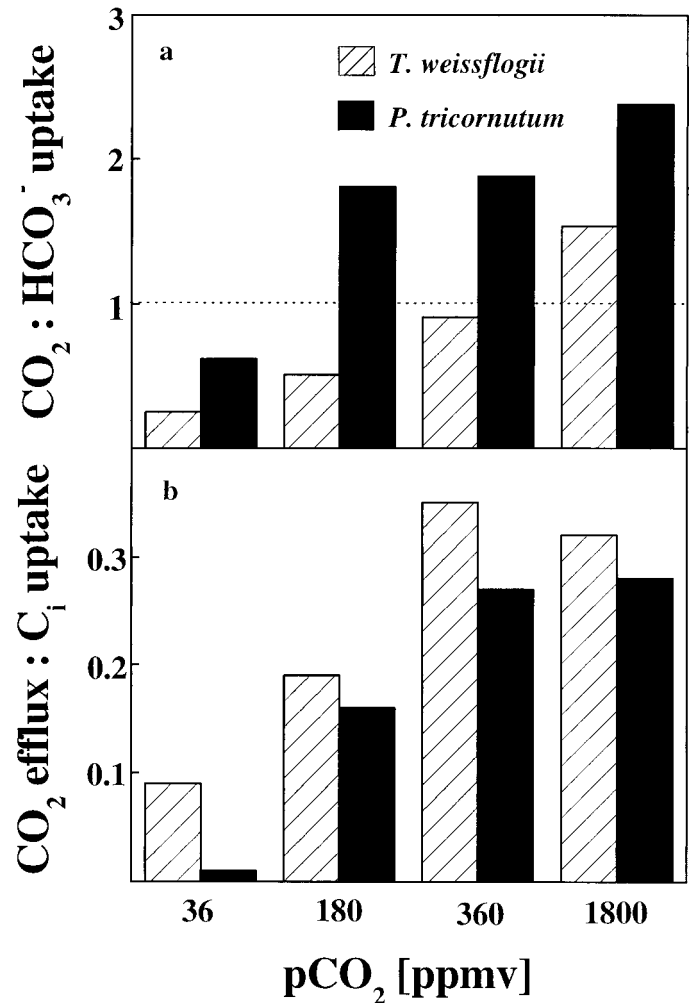


Fig. 8. Ratios of (a) gross  $\text{CO}_2:\text{HCO}_3^-$  uptake and (b)  $\text{CO}_2$  efflux: gross  $C_i$  uptake in *P. tricornutum* and *T. weissflogii* acclimated to different  $p\text{CO}_2$ . The dashed line indicates the value at which  $\text{CO}_2$  and  $\text{HCO}_3^-$  are taken up at equal proportions.

and  $\text{CO}_2$  leakage as a function of  $p\text{CO}_2$  bear important implications for the interpretation of stable carbon isotope data. Several models of carbon isotope fractionation ( $\epsilon_p$ ) indicate that the key determinants are the isotopic composition  $\delta^{13}\text{C}$  of the inorganic carbon source and the ratio of  $C_i$  influx to  $C_i$  efflux (Francois et al. 1993; Laws et al. 1995; Rau et al. 1996; Burkhardt et al. 1999). We applied the model of Burkhardt et al. (1999) to calculate isotope fractionation as a function of  $[\text{CO}_{2,\text{aq}}]$  from our estimates of  $\text{CO}_2:\text{HCO}_3^-$  uptake ratios and  $\text{CO}_2$  leakage (Fig. 8) according to

$$\epsilon_p = a\epsilon_3 + \epsilon_2 F_{-1}/F_r \quad (3)$$

In this equation,  $a$  is the fractional contribution of  $\text{HCO}_3^-$  uptake to total  $C_i$  uptake  $F_r$ ,  $F_{-1}$  is  $\text{CO}_2$  efflux,  $\epsilon_3$  is the equilibrium fractionation between  $\text{CO}_2$  and  $\text{HCO}_3^-$  (assumed to be  $-10\%$ ), and  $\epsilon_2$  is the enzymatic fractionation during carbon fixation (assumed to be  $27\%$ ).

To evaluate whether our calculations of  $\epsilon_p$  provide reasonable trends, we compared model results with  $\epsilon_p$  values

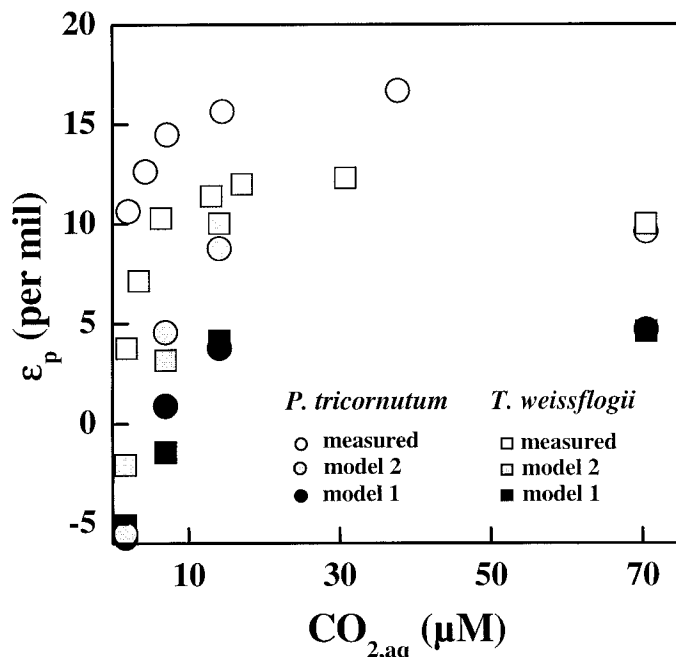


Fig. 9. Stable carbon isotope fractionation ( $\epsilon_p$ ) as a function of CO<sub>2</sub> concentration. Open symbols indicate measured data for *P. tricornutum* (Burkhardt et al. 1999) and *T. weissflogii* (U. Riebesell unpubl. data). Black symbols (model 1) represent  $\epsilon_p$  values according to an isotope fractionation model (Burkhardt et al. 1999) that uses parameters obtained in this study. Gray symbols (model 2) indicate the effect of an increase in CO<sub>2</sub> efflux by a factor of two on  $\epsilon_p$ . Further explanations are provided in the text.

measured in dilute batch cultures under nutrient-replete conditions in continuous light for *P. tricornutum*, strain CCAP1052/1A (Burkhardt et al. 1999) and *T. weissflogii* (U. Riebesell unpubl. data) (Fig. 9). As indicated by relatively constant fractionation at higher CO<sub>2</sub> concentrations and an increase in  $\epsilon_p$  by  $\sim 9\%$  over the experimental CO<sub>2</sub> range, our model calculations yield similar trends as direct measurements of  $\epsilon_p$ . However, we observed a systematic offset by  $\sim 7\%$  in *T. weissflogii* and by  $\sim 12\%$  in *P. tricornutum* between measured and calculated  $\epsilon_p$ . One should keep in mind that the isotope fractionation model can only serve as an approximation of  $\epsilon_p$  because it neglects cell compartmentation, which is likely to have an impact on isotope fractionation (Burkhardt et al. 1999). Because the model is sensitive to photosynthetic rates, higher growth rates in the aerated cultures may also account for some of this discrepancy. Another important parameter in the calculation of  $\epsilon_p$  is an accurate estimate of C<sub>i</sub> fluxes. As discussed above, both an underestimate of CO<sub>2</sub> efflux and the neglect of HCO<sub>3</sub><sup>-</sup> efflux may strongly influence flux estimates. In analogy to our calculation of C<sub>i</sub> fluxes, HCO<sub>3</sub><sup>-</sup> efflux is considered negligible in the isotope fractionation model. Therefore, it is difficult to assess how additional HCO<sub>3</sub><sup>-</sup> efflux might affect  $\epsilon_p$ . An increase in the relative contribution of HCO<sub>3</sub><sup>-</sup> uptake to total uptake causes a decrease in  $\epsilon_p$  that would make the discrepancy between calculated and measured fractionation even larger. On the other hand, higher  $\epsilon_p$  values could the-

oretically be achieved if massive C<sub>i</sub> efflux occurred at the same time. Much of the discrepancy between measurement and model calculations could also be explained in the absence of HCO<sub>3</sub><sup>-</sup> efflux: higher rates of CO<sub>2</sub> efflux would lead to higher CO<sub>2</sub>:HCO<sub>3</sub><sup>-</sup> uptake ratios and to higher leakage values in Fig. 8. An increase in either parameter, in turn, would yield larger fractionation. As indicated in Fig. 9, an increase in CO<sub>2</sub> efflux by a factor of two would cause an increase in  $\epsilon_p$  by  $\sim 5\%$ .

These considerations indicate that a systematic underestimate in CO<sub>2</sub> efflux may, in part, be responsible for the low  $\epsilon_p$  values calculated by the fractionation model. Nevertheless, we were able to produce realistic trends in the  $\epsilon_p$  versus [CO<sub>2, aq</sub>] relationship on the basis of our C<sub>i</sub> flux data, which provides evidence that the frequently observed decrease in  $\epsilon_p$  in response to diminishing CO<sub>2</sub> supply may be the combined result of the gradual induction of HCO<sub>3</sub><sup>-</sup> uptake and a decrease in CO<sub>2</sub> leakage.

In conclusion, results from this study indicate that, in spite of species-specific differences in certain aspects of C<sub>i</sub> acquisition, the overall responses of the two marine diatoms tested here to changes in CO<sub>2</sub> supply closely resemble those reported for freshwater and halotolerant microalgae (Badger et al. 1994; Palmqvist et al. 1994; Amoroso et al. 1998). Highly efficient and inducible uptake mechanisms for both CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> demonstrate the capacity of these organisms to maintain high photosynthetic rates in response to large variations in the marine carbonate system. Even at CO<sub>2</sub> concentrations exceeding current levels in ocean surface waters by a factor of five, CO<sub>2</sub> uptake is supplemented by direct uptake of HCO<sub>3</sub><sup>-</sup>. On the other hand, our results clearly indicate that CO<sub>2</sub> remains an important substrate for photosynthesis even at CO<sub>2</sub> concentrations well below CO<sub>2</sub> levels typically encountered in the ocean. Under the assumption that CO<sub>2</sub> uptake requires less energy than HCO<sub>3</sub><sup>-</sup> uptake, the advantage of a highly efficient CO<sub>2</sub> uptake mechanism may be to facilitate photosynthetic performance in a light-limited environment.

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