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CO_2 and HCO_3^- uptake in marine diatoms acclimated to different CO_2 concentrations

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Abstract

Rates of cellular uptake of CO_2 and HCO_3^- during steady-state photosynthesis were measured in the marine diatoms *Thalassiosira weissflogii* and *Phaeodactylum tricornutum,* acclimated to CO_2 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_2 availability. Both species responded to diminishing CO_2 supply with an increase in eCA and iCA activity. In *P. tricornutum,* eCA activity was close to the detection limit at higher CO_2 concentrations. Simultaneous uptake of CO_2 and HCO_3^- was observed in both diatoms. At air-equilibrated CO_2 levels (360 ppmv), *T. weissflogii* took up CO_2 and HCO_3^- at approximately the same rate, whereas CO_2 uptake exceeded HCO_3^- uptake by a factor of two in *P. tricornutum.* In both diatoms, $CO_2:HCO_3^-$ uptake ratios progressively decreased with decreasing CO_2 concentration, whereas substrate affinities of CO_2 and HCO_3^- uptake increased. Half-saturation concentrations were always ≤ 5 μM CO_2 for CO_2 uptake and <700 μM HCO_3^- for HCO_3^- uptake. Our results indicate the presence of highly efficient uptake systems for CO_2 and HCO_3^- 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_3^- concentrations (~2 mM) but low and variable concentrations of molecular dissolved CO_2 [$CO_{2,aq}$] (~5–25 μM). Variation in [$CO_{2,aq}$] 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_2 content. On longer timescales, rising CO_2 concentrations in the upper layers of the ocean are expected in response to the present increase in atmospheric CO_2 partial pressure (pCO_2; Houghton et al. 1996). Because these changes in [$CO_{2,aq}$] are always accompanied by changes in pH, concentrations of HCO_3^- vary much less because of concomitant shifts in the relative proportions of the inorganic carbon (C_i) species.

The response of phytoplankton growth to changes in CO_2 supply is largely determined by the mechanism of C_i uptake. Several studies indicate that both CO_2 and HCO_3^- 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_2 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_3^- 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_3^- utilization occurs either by direct active uptake or by extracellular catalytic conversion of HCO_3^- to CO_2 in the presence of carbonic anhydrase (CA), with CO_2 entering the cell (Badger et al. 1998; Kaplan et al. 1998; Sültemeyer et al. 1998). In the case of direct HCO_3^- uptake, cellular growth is expected to be more or less unaffected by external CO_2. In contrast, CA-mediated utilization of HCO_3^- accelerates CO_2 formation from HCO_3^- but has no effect on CO_2 equilibrium concentrations at the cell surface. Therefore, the presence of extracellular CA does not prevent phytoplankton from being affected by changes in extracellular [$CO_{2,aq}$].

The proportion at which CO_2 and HCO_3^- are taken up and the extent to which C_i uptake is affected by changes in CO_2 supply are still poorly quantified in marine microalgae. Evidence for HCO_3^- utilization is often provided by methods that do not differentiate between CA-catalyzed HCO_3^- use (with subsequent CO_2 uptake) and direct HCO_3^- uptake across the plasmalemma (e.g., Burns and Beardall 1987;

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Korb et al. 1997). Other studies infer direct HCO_3^- transport from pH drift experiments and the inhibition of light-dependent C_i utilization by 4′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_i fluxes can be derived from this approach. Separate quantification of CO_2 and HCO_3^- 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_2 and HCO_3^- during light-dependent C_i uptake to differentiate between CO_2 and HCO_3^- 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_2 and HCO_3^- during steady-state photosynthesis in relation to external CO_2 supply in two marine diatoms by use of membrane inlet mass spectrometry. We used this approach to determine substrate preferences for CO_2 and HCO_3^- uptake as well as possible shifts in carbon source and C_i uptake kinetics in cells acclimated to different $[CO_{2,aq}]$. 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 ^{18}O exchange from doubly labelled $^{13}C^{18}O_2$ 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^{-2} s^{-1}. Different CO_2 partial pressures (pCO_2) in the medium were achieved by a continuous flow of 0.2-μm-filtered air containing 36, 180, 360, or 1,800 ppmv CO_2. Because the culture medium was not buffered artificially, an increase from 36 to 1,800 ppmv CO_2 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^{-1}.

For measurements of CA activity or C_i 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_2-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_i 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, $[CO_{2,aq}]$, and $[HCO_3^-]$—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_2 ($[CO_{2,aq}]$) and HCO_3^- ($[HCO_3^-]$) 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 ^{18}O from doubly labelled $^{13}C^{18}O_2$ to water caused by several hydration and dehydration steps of CO_2 and HCO_3^- (Silverman 1982; Badger and Price 1989; Sültemeyer et al. 1990). Doubly labelled CO_2 was prepared from $H_2{}^{18}O$ (98 atom% enrichment) and $NaH^{13}CO_3$ (99 atom% enrichment), both purchased from Chemotrade (Leipzig, Germany). $NaH^{13}CO_3$ was added to $H_2{}^{18}O$ (1 M C_i final concentration) under N_2 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_2. The reaction sequence of ^{18}O loss from $^{13}C^{18}O^{18}O$ (m/z = 49) with the intermediate product $^{13}C^{18}O^{16}O$ (m/z = 47), and the end product $^{13}C^{16}O^{16}O$ (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 ^{18}O in doubly labelled CO_2 is an irreversible reaction, because $H_2{}^{18}O$ produced is infinitely diluted with $H_2{}^{16}O$.

For determination of CA activity, relative ^{18}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-

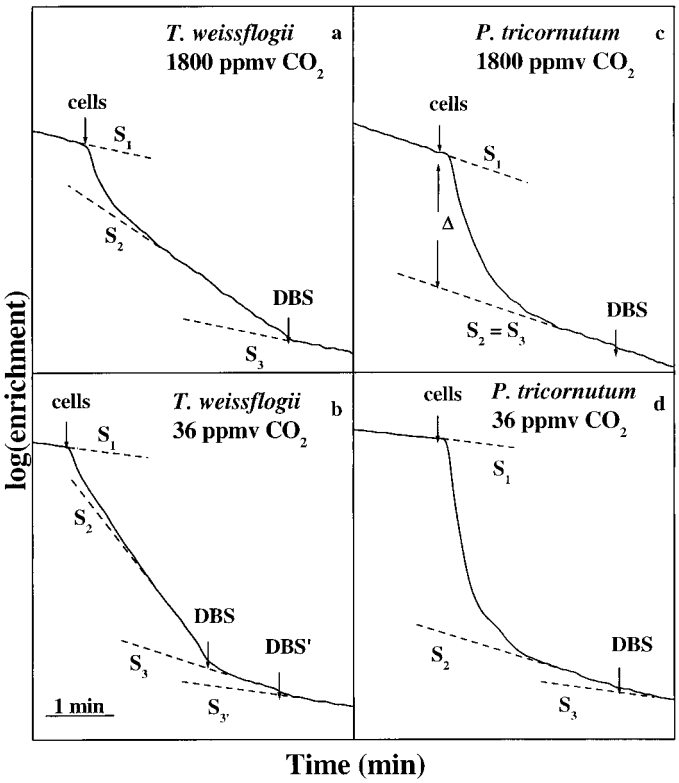

Fig. 1. In vivo measurements of CA activity based on ^{18}O exchange between doubly labelled CO_2 ($^{13}C^{18}O^{18}O$, $m/z = 49$) and water. The reaction sequence of ^{18}O loss from $m/z = 49$ to $m/z = 45$ with the intermediate product $m/z = 47$ was monitored by membrane inlet mass spectrometry and is reported as log(enrichment) according to Eq. 1. The initial slope (S_1) represents ^{18}O exchange during the uncatalyzed hydration/dehydration reaction between CO_2 and HCO_3^-. The difference between S_2 and S_1 indicates ^{18}O exchange in the presence of eCA. eCA activity is calculated according to Eq. 2. S_3 and S_3' represent ^{18}O exchange upon the addition of 50 μM or 100 μM DBS (an inhibitor of eCA), respectively. The drop (Δ) in log(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. tricornutum,* (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°C). 10 μl of $NaH^{13}C^{18}O_3$ solution were added to achieve 1 mM final concentration. After chemical equilibrium was reached (initial slope, S_1), 50–100 μl of washed cells (resuspended pellet) were added to a final Chl *a* concentration of 1–3 μg ml^{-1}. To avoid interference of 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}O exchange, which is represented by slope S_2 in Fig. 1. Intracellular CA activity was estimated from the rapid decline in log(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 CO_2 or during steady-state depletion of ^{18}O due to eCA activity. The resulting rates of log(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}O depletion. Furthermore, DBS concentrations were determined at which complete inhibition of eCA activity was achieved. This inhibition is a prerequisite for measurements of CO_2 uptake and HCO_3^- transport, which rely on the relatively slow spontaneous interconversion between CO_2 and HCO_3^-.

Determination of CO_2 uptake, 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 O_2 and CO_2 during consecutive light (4 min) and dark (3 min) intervals.

To estimate CO_2 and 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 CO_2-free f/2 medium, buffered with 50 mM HEPES (pH 8.0, 15°C) within 10 min after centrifugation, and the cuvette was closed immediately. Chl *a* concentrations in the cuvette were 1–3 μg ml^{-1}. DBS was added prior to cell addition at concentrations of 50–100 μM (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 μmol photons m^{-2} s^{-1} incident PFD and a CO_2 concentration of typically <1 μM ($C_i < 100$ μM). Between the subsequent light : dark cycles, known amounts of C_i were added to measure uptake rates as a function of CO_2 and HCO_3^- concentrations.

Rates of O_2 consumption in the dark and 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 O_2 fluxes into C_i fluxes. Gross CO_2 uptake was calculated from the steady-state rate of CO_2 depletion at the end of the light period and the initial rate of CO_2 generation immediately after the light was turned off. This calculation is based on the assumption that the rate of diffusive CO_2 efflux from a cell in the light represents the rate of CO_2 efflux during the first seconds of the dark phase.

HCO_3^- uptake was calculated from net C_i fixation, corrected for measured steady-state depletion of CO_2 at the end of the light period and for CO_2/HCO_3^- interconversion in the medium. This calculation refers to net HCO_3^- uptake. As will be discussed below, gross HCO_3^- uptake equals net HCO_3^- only if diffusive efflux of HCO_3^- is negligible. In calculations, the required pseudo first-order rate constant k_2 (formation of CO_2 from HCO_3^-) was experimentally determined from the initial slope of CO_2 evolution from HCO_3^-

after injection of known amounts of HCO_3^- into CO_2-free buffered medium. Several independent measurements yielded a mean value (± 1 SD, $n = 10$) of $k_2 = 8.9$ (± 1.1) $\times 10^{-3}$ min^{-1}. The k_1 rate constant (formation of HCO_3^- from CO_2) was calculated from the product of k_2 and the ratio of CO_2 and HCO_3^- 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_2 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_2 were used in these experiments. Changes in the O_2 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_i uptake measurements. CO_2 concentrations in the medium were 1 and 13 μM. During steady-state photosynthesis in the light, DBS (100 μM final concentration) was injected into the closed cuvette to inhibit eCA activity. In additional tests, 60 μg of commercially available CA from bovine erythrocytes (Sigma Chemical Co., Munich, Germany; 4090 W-A units mg^{-1} protein) was added during measurements of O_2 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 μM (Fig. 1a,d) or 100 μM (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 ^{18}O depletion from $^{13}C^{18}O_2$ 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_2 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_2, eCA activity was highest, but it decreased progressively with increasing pCO_2. Over the experimental CO_2 range, eCA activity varied by a factor of ~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 $^{13}C^{18}O^{18}O$ into the cells, in which doubly labelled CO_2 is initially absent. In the presence of iCA, ^{18}O loss from CO_2 of $m/z = 49$ is

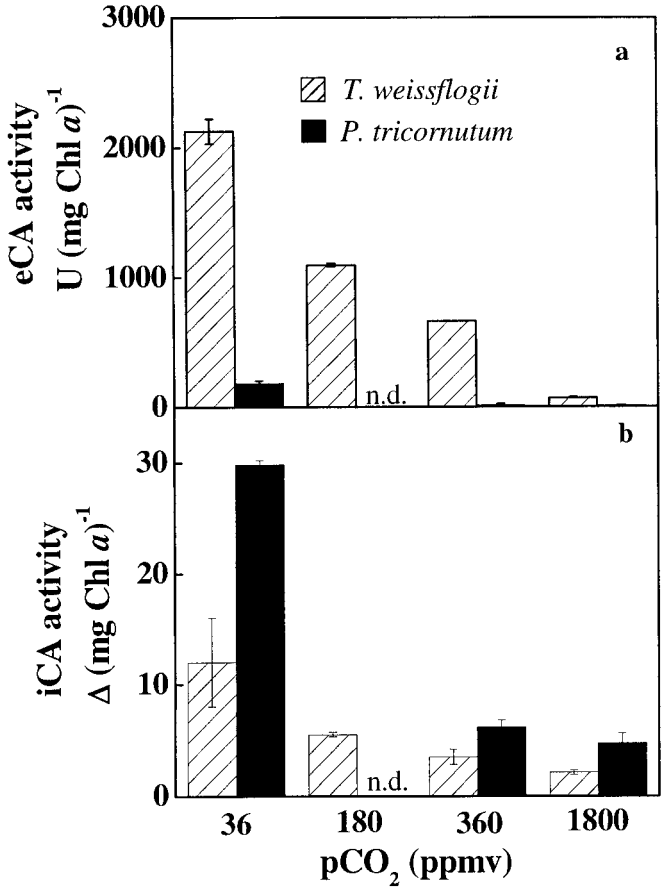

Fig. 2. Chl *a*–specific activities of (a) eCA and (b) iCA in *T. weissflogii* and *P. tricornutum* as a function of pCO_2 in the culture medium, to which the cells have been acclimated. n.d. = not determined.

catalyzed inside the cell, followed by diffusion of the newly generated CO_2 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_2, the initial decline in log(enrichment) was almost 50% inhibited by 10 μM of the membrane permeable CA-inhibitor ethoxyzolamide, whereas 200 μM 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_2 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 ^{18}O from the medium in the presence of cells. Subsequently, iCA-mediated ^{18}O depletion from CO_2 in the medium solely depends on the balance between extracellular dehydration of ^{18}O-containing HCO_3^- and the rate of CO_2 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 Δ as the drop in log(enrichment) when S_2 is extrapolated to the time of cell addition (Fig. 1). Because Δ is only determined by (1) the rate of diffusive CO_2 transport across cell membranes, (2)

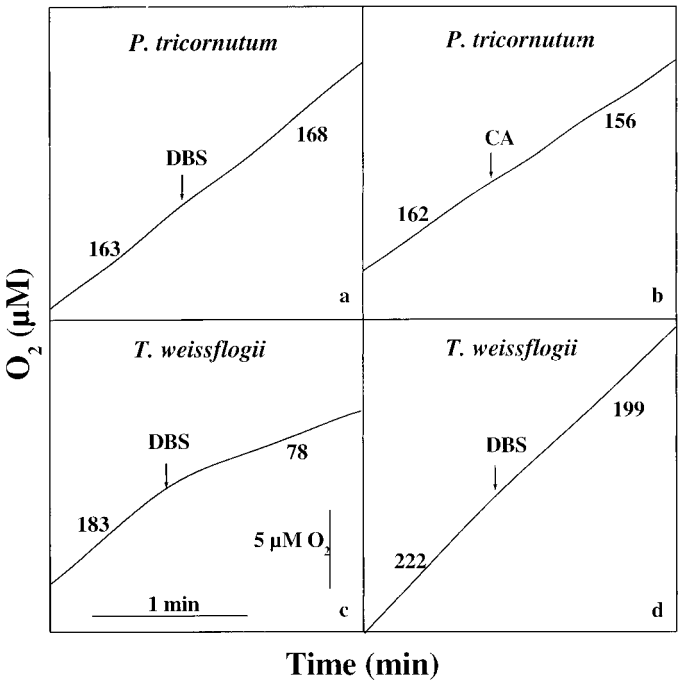

Fig. 3. Effect of the addition of the eCA-inhibitor DBS or commercially available CA on photosynthetic O_2 evolution in (a, b) *P. tricornutum* acclimated to 36 ppmv CO_2 and (c, d) *T. weissflogii* acclimated to 180 ppmv CO_2. The values close to the slopes indicate Chl *a*–specific rates [μmol (mg^{-1} Chl *a*)$^{-1}$ h^{-1}] before and after addition of DBS/CA. Shown are typical experiments out of four to five independent replicates.

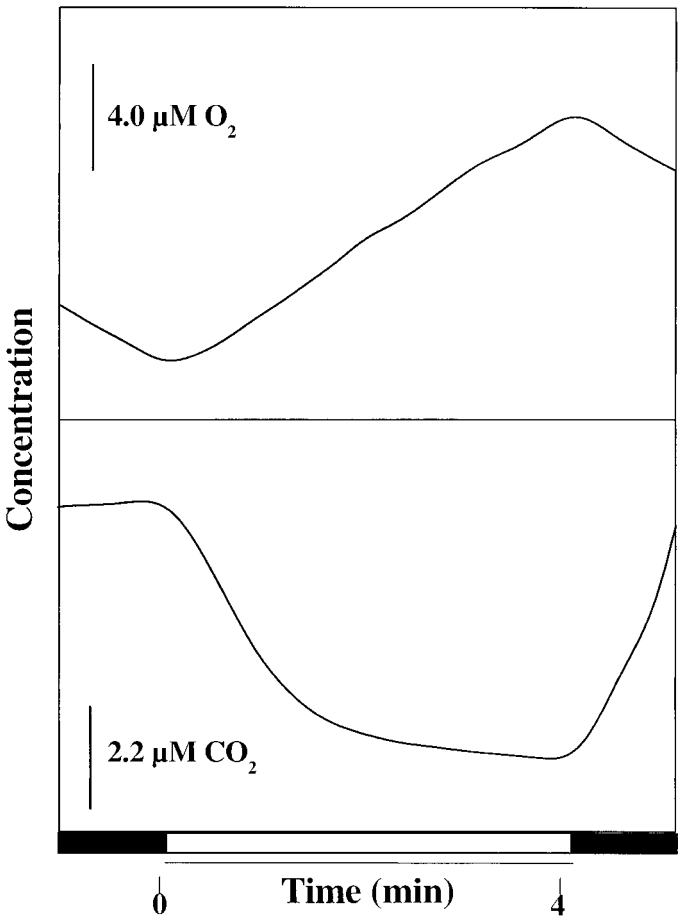

Fig. 4. Continuous measurements of O_2 and CO_2 concentrations used to estimate CO_2 uptake, HCO_3^- uptake, and photosynthetic O_2 evolution. Data shown in this figure were obtained with *T. weissflogii*, acclimated to 1,800 ppmv CO_2. The white bar at the bottom indicates a 4-min light period.

intracellular ^{18}O depletion catalyzed by iCA, and (3) the rate of extracellular ^{18}O depletion (spontaneous and eCA-catalyzed), correction for S_2 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_2 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_2 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_2 in the cultures. In both species we observed a significant decline in iCA activity with increasing pCO_2, reflected in an approximately sixfold change over the experimental CO_2 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_2 even at $[CO_{2,aq}] = 1$ μM in the assay medium (Fig. 3a), which represents the CO_2 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_2, inhibition of eCA caused a decline in net photosynthesis to less than half of the original value at $[CO_{2,aq}] = 1$ μM (Fig. 3c). When incubated at 13 μM of CO_2, the addition of DBS still affected photosynthesis, but the rate decreased by only ~10% (Fig. 3d).

CO_2 uptake, HCO_3^- uptake, and net photosynthesis—As indicated by a typical time course of changes in O_2 and CO_2 concentrations (Fig. 4), the transition from dark to light was accompanied by an immediate onset of photosynthetic O_2 production and CO_2 consumption. After ~3 min, the rate of decrease in CO_2 concentration in the cuvette reached steady state. When the light was turned off, photosynthetic O_2 production ceased immediately and was replaced by respiratory O_2 consumption. The initial CO_2 efflux in the dark caused the CO_2 concentration to increase rapidly.

Simultaneous uptake of CO_2 and HCO_3^- during steady-state photosynthesis was observed in both diatoms (Fig. 5,

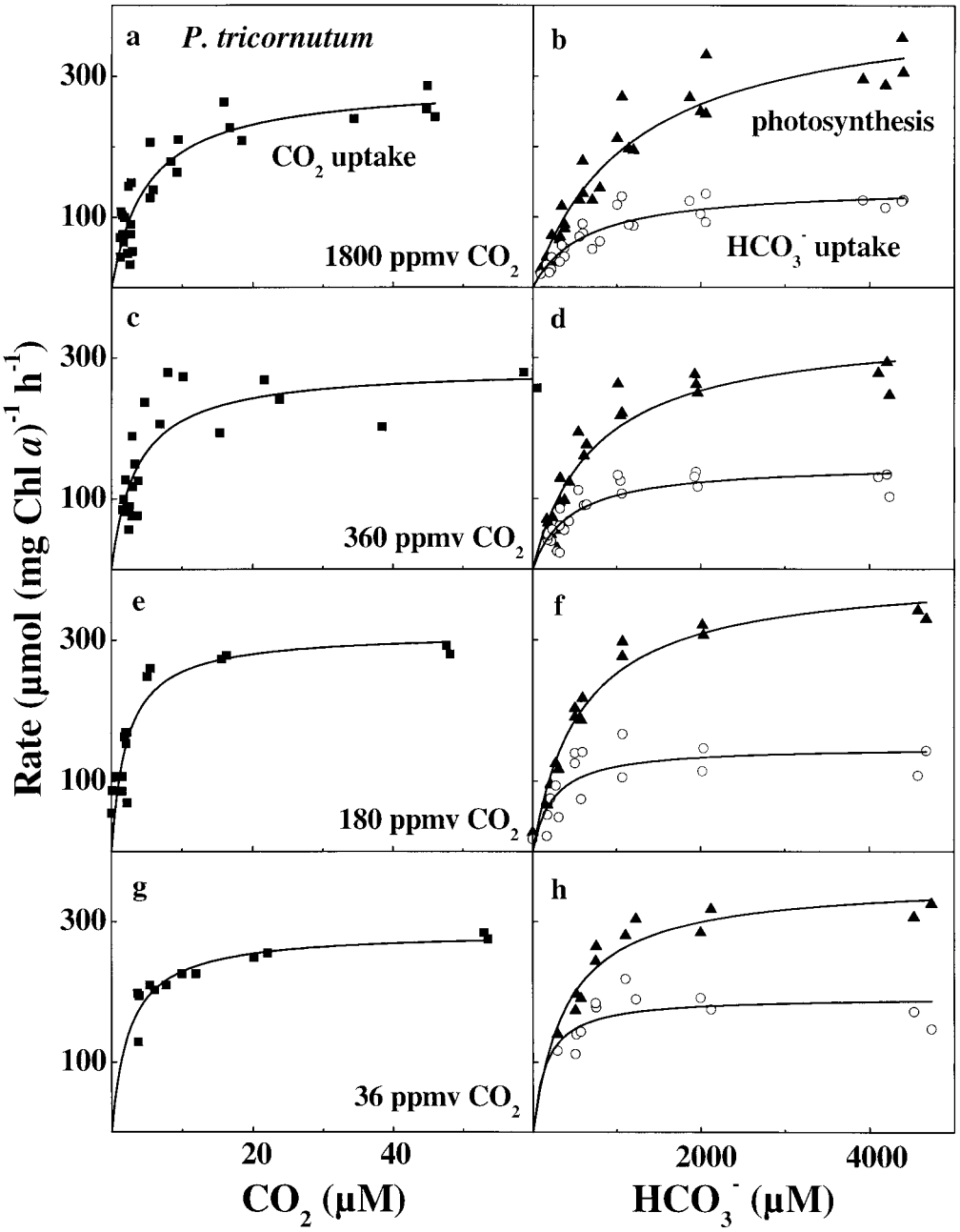

Fig. 5. *P. tricornutum.* Chl *a*–specific rates of gross CO_2 uptake (squares), HCO_3^- uptake (circles), and net photosynthesis (triangles) as a function of CO_2 or HCO_3^- concentration in the assay medium. Prior to the measurements, cultures were acclimated to (a, b) 1,800 ppmv; (c, d) 360 ppmv; (e, f) 180 ppmv; or (g, h) 36 ppmv of CO_2 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_2 on uptake kinetics existed. Maximum rates (V_{max}) and half-saturation concentrations ($K_{1/2}$) of inorganic carbon uptake and photosynthetic O_2 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_2 uptake, HCO_3^- uptake, and net O_2 evolution were largely unaffected by acclimation to different pCO_2 (Figs. 5 and 7b). In general, rates of CO_2 uptake were approximately twice the rates of HCO_3^- uptake, which indicates that CO_2 was the preferred substrate for inorganic carbon uptake in this species. In *T. weissflogii,* maximum rates of HCO_3^- uptake and net O_2 evolution also remained largely unaffected by acclimation to a wide range of pCO_2 (Figs. 5 and 7a). A significant decrease

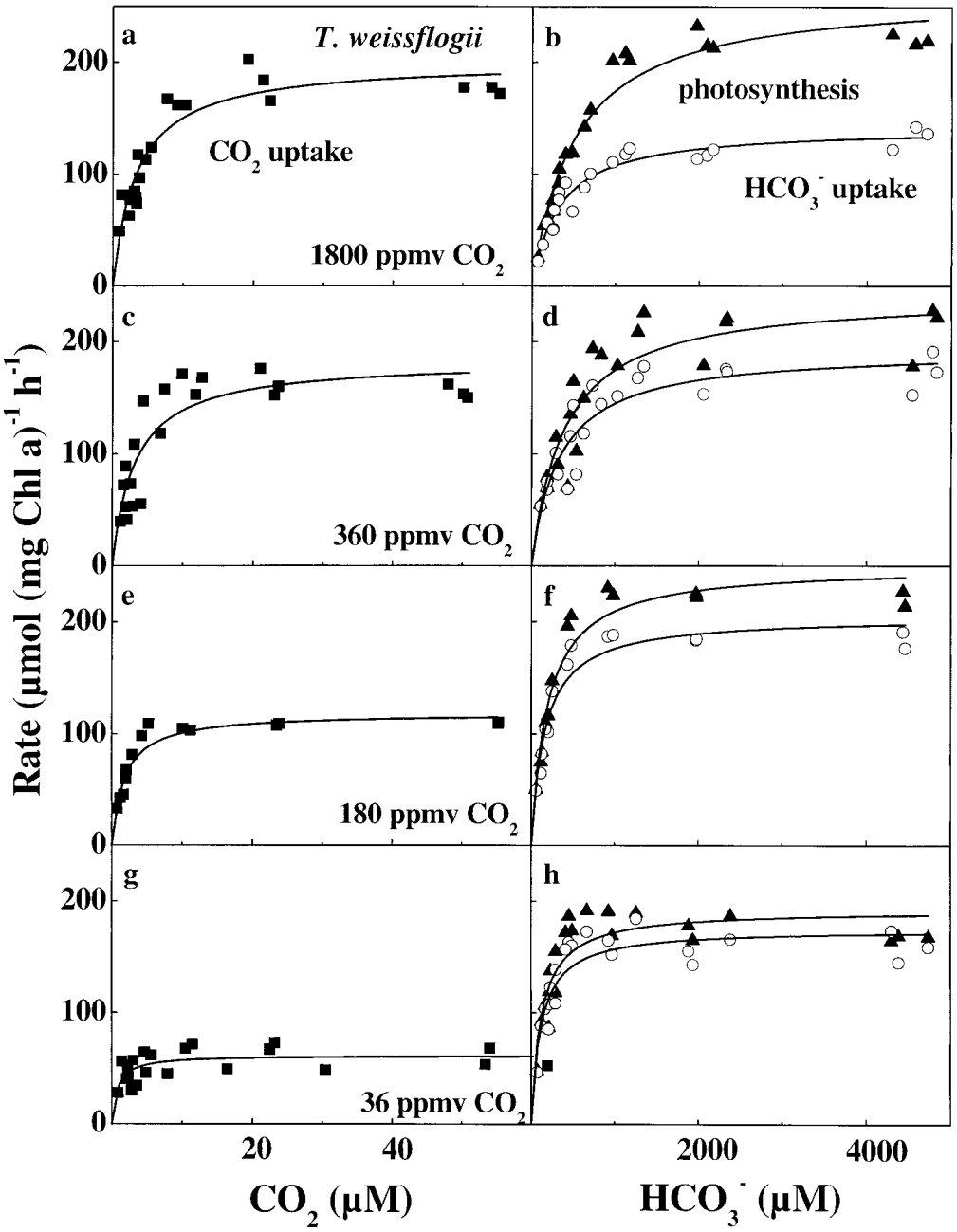

Fig. 6. *T. weissflogii.* Chl *a*–specific rates of gross CO_2 uptake (squares), HCO_3^- uptake (circles), and net photosynthesis (triangles) as a function of CO_2 or HCO_3^- concentration in the assay medium. Prior to the measurements, cultures were acclimated to (a, b) 1,800 ppmv; (c, d) 360 ppmv; (e, f) 180 ppmv; or (g, h) 36 ppmv of CO_2 for at least 48 h. Curves were obtained from a Michaelis-Menten fit.

in V_{max} was only observed for HCO_3^- uptake at the highest pCO_2 and for photosynthetic O_2 evolution at the lowest pCO_2. Unlike *P. tricornutum*, however, V_{max} of gross CO_2 uptake continuously decreased with decreasing pCO_2. This indicates that CO_2 was preferentially taken up by cells acclimated to 1,800 ppmv CO_2, whereas HCO_3^- uptake dominated in cells grown at 36 ppmv CO_2.

An increase in substrate affinities of carbon uptake upon acclimation to decreasing CO_2 supply was observed in both diatoms, as was indicated by a decrease in $K_{1/2}(CO_2)$ of CO_2 uptake and in $K_{1/2}(HCO_3^-)$ of HCO_3^- uptake with decreasing pCO_2 in the cultures (Fig. 7c,d). Half-saturation of CO_2 uptake generally occurred at $[CO_{2,aq}] \leq 5$ μM in both species. Half-saturation concentrations of HCO_3^- uptake were slightly lower in *T. weissflogii* than in *P. tricornutum* but were always <700 μM.

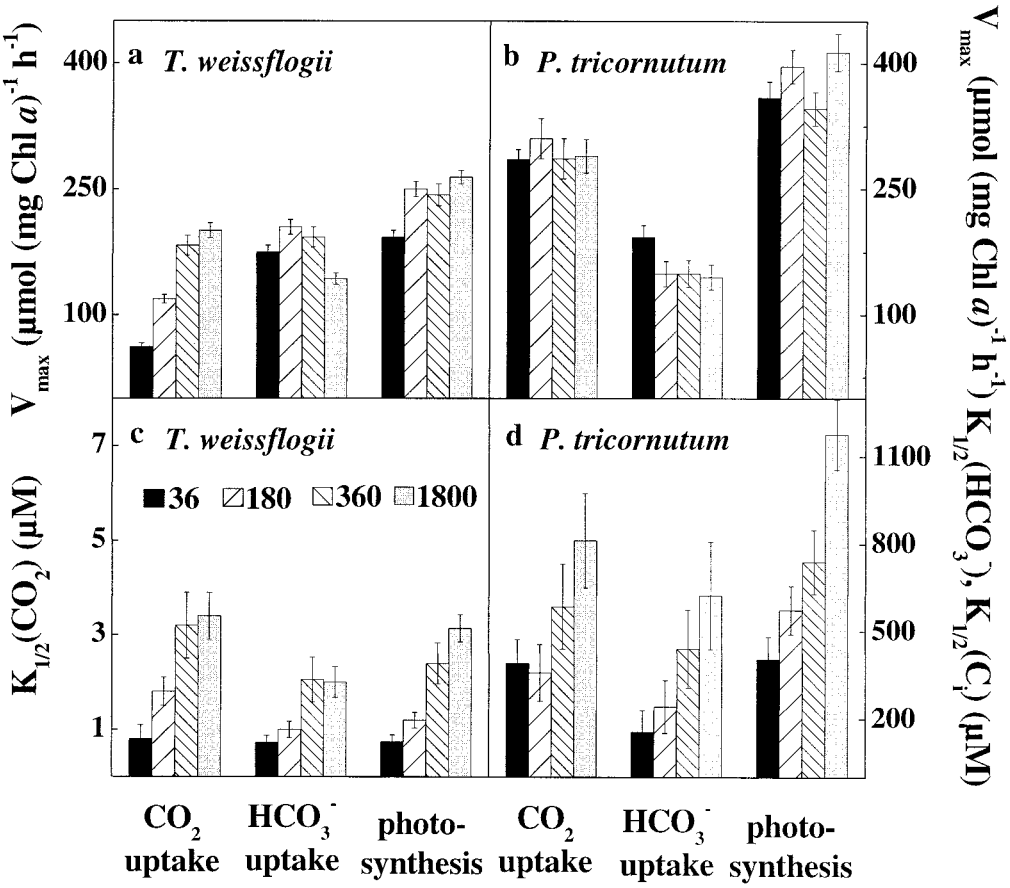

Fig. 7. V_{max} and $K_{1/2}$ of gross CO_2 uptake, HCO_3^- uptake, and photosynthetic O_2 evolution for (b, d) *P. tricornutum* and (a, c) *T. weissflogii.* Kinetic parameters were calculated from a Michaelis-Menten fit to the combined data of several independent measurements. Error bars indicate ±1 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_2 were harvested at a higher Chl *a* concentration (250 μg l^{-1}). In these cultures, the rate of CO_2 uptake exceeded the rate of CO_2 supply, so that CO_2 concentrations were no longer at equilibrium with the air stream. Consequently, $[CO_{2,aq}]$ dropped to 0.01 μM, HCO_3^- decreased to 117 μM, and pH increased to 10.1. Compared with experiments at 36 ppmv CO_2 under equilibrium conditions, V_{max} of CO_2 and HCO_3^- uptake as well as $K_{1/2}(CO_2)$ of CO_2 uptake were largely unaffected by the further C_i depletion (Table 1). On the other hand, we observed a decline in $K_{1/2}(HCO_3^-)$ of HCO_3^- uptake by a factor of 10 under these conditions.

Table 1. *P. tricornutum.* V_{max}, $K_{1/2}(CO_2)$, and $K_{1/2}(HCO_3^-)$ (±1 SD) of CO_2 uptake and HCO_3^- uptake. All cultures were aerated with 36 ppmv CO_2. Cultures growing at equilibrium with CO_2 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_2 consumption exceeded the rate of CO_2 supply.

	CO_2 supply = CO_2 consumption, nonbuffered	CO_2 supply < CO_2 consumption	
		Nonbuffered	Buffered at pH 8.0
CO_2 uptake			
V_{max}	285 (12)	282 (16)	256 (12)
$K_{1/2}(CO_2)$	2.4 (0.5)	1.7 (0.4)	1.5 (0.3)
HCO_3^- uptake			
V_{max}	193 (14)	201 (10)	185 (9)
$K_{1/2}(HCO_3^-)$	155 (74)	12 (8)	15 (15)

Similar kinetic constants were obtained in *P. tricornutum* cultivated at 36 ppmv CO_2 in the presence of buffer (20 mM HEPES) at a constant pH of 8.0 (Table 1). Equilibrium concentrations of CO_2 and HCO_3^- were 1.4 and 116 μM in the buffered cultures, but lower concentrations are, again, expected when cells are harvested at 250 μg l^{-1} Chl *a.* In both buffered and nonbuffered cultures, these extremely low concentrations of inorganic carbon yielded values of $K_{1/2}(CO_2)$ = 1.5–1.7 μM for CO_2 uptake and $K_{1/2}(HCO_3^-)$ = 12–15 μM for HCO_3^- 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_2 and HCO_3^- during photosynthesis even after growth at high CO_2 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_2 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_2 uptake to total C_i uptake in *P. tricornutum* compared with the greater proportion of HCO_3^- uptake in *T. weissflogii*. Furthermore, the maximum rate of CO_2 uptake in *P. tricornutum* remains largely unaffected by variable pCO_2, whereas it decreases upon CO_2 depletion in *T. weissflogii*. Changes in V_{max} 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_i 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_2 levels of 360 ppmv or higher and remained low even in the lowest pCO_2 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_2 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_i 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 catalytical conversion of HCO_3^- to CO_2. In contrast, eCA activity appears to be crucial in other strains of *P. tricornutum*, as indicated by a significant decrease in photosynthetic CO_2 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_3^- appears to be the dominant C_i species taken up by the cells, especially at low CO_2 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_2 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_2, this effect was large at low $[CO_{2,aq}]$ (1 μM) but small at higher $[CO_{2,aq}]$ (13 μM). At 1 μM of CO_2, corresponding to ~90 μM of HCO_3^- at pH 8.0, cells acclimated to 180 ppmv CO_2 exhibited similar rates of CO_2 and HCO_3^- uptake. This indicates that, under limiting C_i concentrations, CO_2 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_2 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_2 and thus on the diffusive properties of algal membranes. Therefore, it is reasonable to conclude that both species respond to a decrease in pCO_2 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_2 across the plasmalemma in the smaller species *P. tricornutum*.

Prior to the discussion of C_i 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_i fluxes is provided by Badger et al. (1994) and by Kaplan and Reinhold (1999). Critical assumptions that may affect C_i flux estimates concern the quantification of CO_2 efflux and the possibility of HCO_3^- efflux from the cells back to the medium. Quantification of CO_2 efflux assumes that the rate of diffusive CO_2 efflux in the light is well represented by the rate of CO_2 efflux during the first seconds of the subsequent dark phase. According to Badger et al. (1994), this method may underestimate actual rates of CO_2 efflux in eukaryotic microalgae as a result of cell compartmentation. Furthermore, we cannot exclude the possibility that an energy-driven transport of CO_2 out of the cell takes place in the light, leading to an underestimate of CO_2 efflux when it is determined from initial rates of CO_2 efflux during the first sec-

onds of the dark phase. In fact, massive CO_2 efflux originating from HCO_3^- uptake with subsequent intracellular conversion to CO_2 was proposed by Tchernov et al. (1997, 1998) on the basis of experiments with the cyanobacterium *Synechoccus* and with *Nannochloropsis* (Eustigmatophyceae). Any underestimate of CO_2 efflux would imply an underestimate of gross CO_2 uptake rates presented in this study.

Evidence for HCO_3^- efflux has, until now, only been reported for cyanobacteria (e.g., Salon et al. 1996; Kaplan and Reinhold 1999). Whether similar rates of HCO_3^- efflux can be achieved in eukaryotic microalgae, which accumulate intracellular C_i to a lesser degree and impose additional membrane barriers for HCO_3^- efflux, remains to be tested. Interpretation of HCO_3^- efflux is complicated by the use of inhibitors of photosynthetic carbon fixation, such as iodacetamid, which are applied during these measurements. This approach has the advantage that HCO_3^- efflux can be measured while light energy is available for active transport processes. Because of the low permeability of biological membranes for HCO_3^-, any HCO_3^- loss from a cell is likely to involve transport processes other than passive diffusion. On the other hand, evidence for HCO_3^- efflux during inhibition of C_i 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_3^- efflux was detected in *P. tricornutum* in the light (300 μmol photons m^{-2} s^{-1}) when treated with 5 mM iodacetamid (Sültemeyer unpubl. data).

If significant HCO_3^- efflux occurred in our assays, our estimates of HCO_3^- uptake would represent net uptake of HCO_3^-. In other words, the method would still be capable of demonstrating the ability of direct HCO_3^- uptake accompanied by CO_2 uptake as an important additional carbon source in photosynthesis. However, $K_{1/2}(HCO_3^-)$ estimates obtained with this approach (Fig. 7) would be erroneous if large HCO_3^- efflux occurred. As long as we are unable to provide accurate measurements of HCO_3^- efflux during photosynthesis in the absence of inhibitors, the assumptions made in our calculations should be kept in mind when interpreting C_i uptake kinetics.

Our observation of simultaneous CO_2 and HCO_3^- 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_2 and HCO_3^- 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_2 level, half-saturation of CO_2 and HCO_3^- uptake was achieved at concentrations of 3.4–5.0 μM CO_2 and 325–623 μM HCO_3^-. Compared with typical $[CO_{2,aq}]$ of >10 μM and $[HCO_3^-]$ of $\sim$2 mM in ocean surface waters, these results provide evidence for highly efficient CO_2 and HCO_3^- uptake in both diatoms. (2) As indicated by a continuous decrease in $K_{1/2}(CO_2)$ of CO_2 uptake and $K_{1/2}(HCO_3^-)$ of HCO_3^- 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_2 uptake kinetics, $K_{1/2}(CO_2)$ values of photosynthesis range from 1.3 to 5.8 μM CO_2, depending on species and culture conditions. In contrast, the few data available for kinetic properties of the CO_2-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_2 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_i 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_2 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_2 was accompanied by a decrease in pH from 9.1 to 7.6 and a decrease in the $[HCO_3^-]:[CO_{2,aq}]$ ratio from 767 to 32. As a result of the variable $[HCO_3^-]:[CO_{2,aq}]$ ratio, a 50-fold increase in $[CO_{2,aq}]$ led to an increase in $[HCO_3^-]$ by only a factor of two. In this respect, buffered and nonbuffered seawater media differ considerably. Whereas both media aerated at different pCO_2 experience the same variation in $[CO_{2,aq}]$ (1.4–70.5 μM in this study), the variation in $[HCO_3^-]$ 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_i uptake kinetics. However, one experiment with *P. tricornutum,* in which extremely low CO_2 and HCO_3^- concentrations were achieved by photosynthetic C_i consumption (exceeding C_i supply), was performed with both buffered and nonbuffered culture medium. Results indicate that maximum substrate affinity of both CO_2 and HCO_3^- transport systems can be induced regardless of a difference in pH by two units (Table 1). In these experiments, $K_{1/2}(CO_2)$ of CO_2 uptake was only slightly lower than in nonbuffered cultures growing at equilibrium with 36 ppmv CO_2 in the air stream. In contrast, $K_{1/2}(HCO_3^-)$ of HCO_3^- uptake decreased drastically from 155 μM (at equilibrium with 36 ppmv CO_2, $[HCO_3^-] = 1{,}074$ μM) to 12–15 μM (excess C_i consumption, $[HCO_3^-] < 117$ μM HCO_3^-). This indicates that the high-affinity HCO_3^- uptake system of *P. tricornutum* is not fully induced in nonbuffered cultures, even when they grow at equilibrium with 36 ppmv CO_2.

Evidence from studies of cyanobacteria suggests that CO_2 and HCO_3^- transport are separate, independent processes (Espie et al. 1991; Omata et al. 1999) and that HCO_3^- is involved as a primary signal to induce high-affinity HCO_3^- transport (Sültemeyer et al. 1998). Our results are consistent with these observations, although we cannot rule out the possibility that changes in CO_2 supply also affect HCO_3^- acquisition in the two diatoms of this study. If the CO_2 and HCO_3^- 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_2-HCO_3^- interconversion reactions invariable. Therefore, cells suspended in buffered assay medium experience,

in contrast to nonbuffered culture medium, constant $[HCO_3^-]:[CO_{2,aq}]$ ratios during flux measurements at any C_i concentration. Although this approach is appropriate to determine changes in CO_2 and 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 $[HCO_3^-]:[CO_{2,aq}]$ ratios during acclimation, we applied measured uptake kinetics (Fig. 7) to calculate CO_2 and HCO_3^- uptake rates as a function of the actual CO_2 and HCO_3^- concentrations to which the diatoms were exposed in the cultures. These calculations are based on the assumption that CO_2 and 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 CO_2:HCO_3^- uptake ratios at different culture conditions. In both species, we observe a systematic decrease in the CO_2:HCO_3^- uptake ratio in response to a decrease in pCO_2 to which the cultures were acclimated (Fig. 8a). The horizontal line indicates the value at which CO_2 and HCO_3^- are taken up at equal proportions. It becomes evident that *P. tricornutum* prefers CO_2 as a substrate at all but the lowest pCO_2. In contrast, *T. weissflogii* shifts from CO_2 to HCO_3^- as preferred carbon substrate at $pCO_2 \leq 360$ ppmv. These results demonstrate that a decline in CO_2 supply is accompanied by the gradual induction of HCO_3^- uptake, whereas both species favor the uptake of CO_2 if present at high concentrations.

Such a regulatory mechanism raises the questions why marine diatoms, which have the capacity for active HCO_3^- transport, do not rely on the abundant HCO_3^- pool in seawater as the only inorganic carbon source. We suggest that the coexistence of both transport systems with preference for CO_2 uptake at higher CO_2 concentrations is maintained for energetic reasons. Even in the case of active CO_2 transport, its energetic cost is expected to be lower than that of 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 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 CO_2 and that the efflux of HCO_3^- is negligible. In this case, our calculations of C_i leakage (defined as the ratio of CO_2 efflux to total C_i uptake) indicate that ~30% of the inorganic carbon taken up is subsequently lost in both diatoms, when acclimated to $pCO_2 \leq 360$ ppmv (Fig. 8b). In response to a decrease in pCO_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 HCO_3^- taken up by low-CO_2 cells.

Our calculations of variable CO_2:HCO_3^- uptake ratios

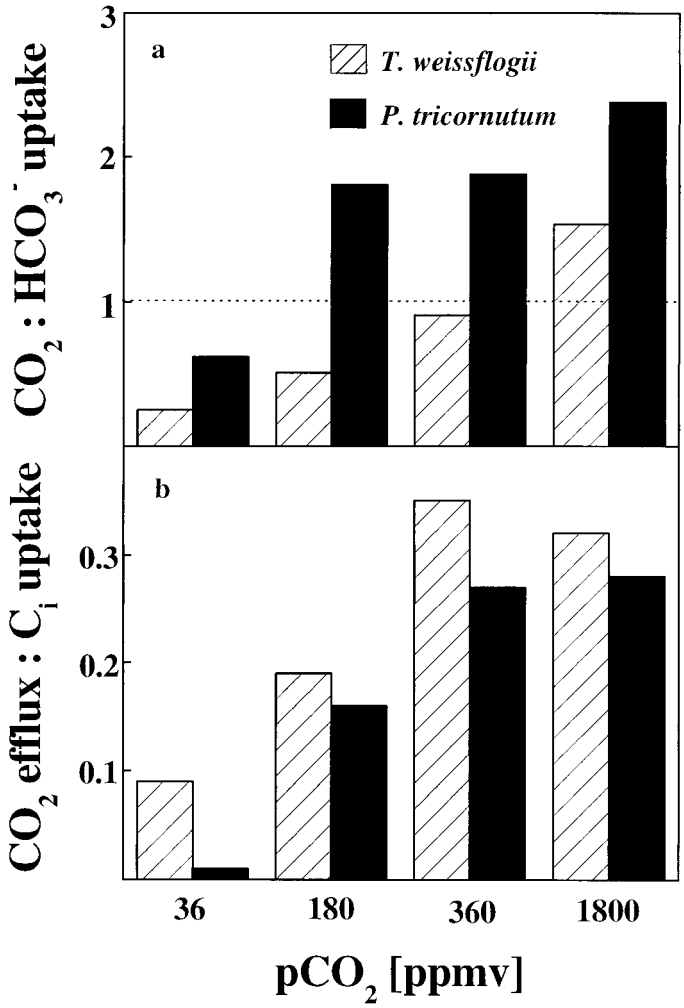

Fig. 8. Ratios of (a) gross CO_2:HCO_3^- uptake and (b) CO_2 efflux:gross C_i uptake in *P. tricornutum* and *T. weissflogii* acclimated to different pCO_2. The dashed line indicates the value at which CO_2 and HCO_3^- are taken up at equal proportions.

and CO_2 leakage as a function of pCO_2 bear important implications for the interpretation of stable carbon isotope data. Several models of carbon isotope fractionation (ε_p) indicate that the key determinants are the isotopic composition $\delta^{13}C$ 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 $[CO_{2,aq}]$ from our estimates of CO_2:HCO_3^- uptake ratios and CO_2 leakage (Fig. 8) according to

$$\varepsilon_p = a\varepsilon_3 + \varepsilon_2 F_{-1}/F_t. \quad (3)$$

In this equation, a is the fractional contribution of HCO_3^- uptake to total C_i uptake F_t, F_{-1} is CO_2 efflux, ε_3 is the equilibrium fractionation between CO_2 and HCO_3^- (assumed to be −10‰), and ε_2 is the enzymatic fractionation during carbon fixation (assumed to be 27‰).

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

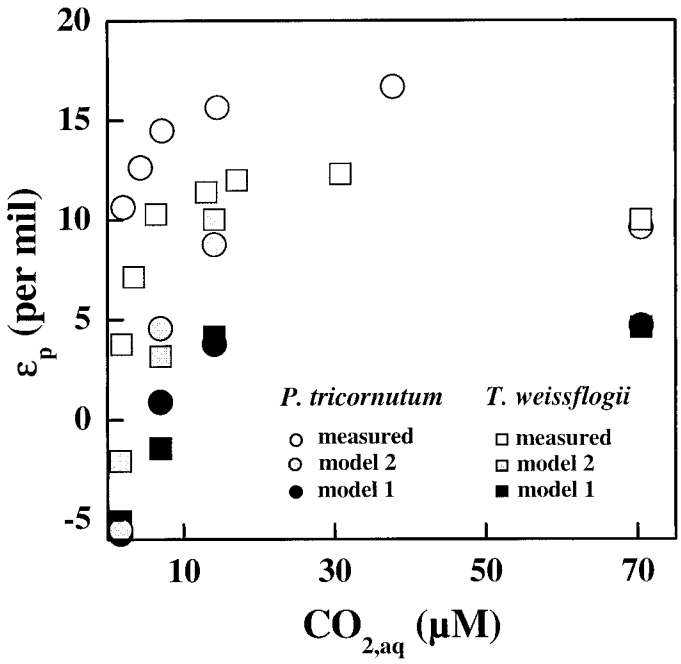

Fig. 9. Stable carbon isotope fractionation (ε_p) as a function of CO_2 concentration. Open symbols indicate measured data for *P. tricornutum* (Burkhardt et al. 1999) and *T. weissflogii* (U. Riebesell unpubl. data). Black symbols (model 1) represent ε_p values according to an isotope fractionation model (Burkhardt et al. 1999) that uses parameters obtained in this study. Gray symbols (model 2) indicate the effect of an increase in CO_2 efflux by a factor of two on ε_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_2 concentrations and an increase in ε_p by ~9‰ over the experimental CO_2 range, our model calculations yield similar trends as direct measurements of ε_p. However, we observed a systematic offset by ~7‰ in *T. weissflogii* and by ~12‰ in *P. tricornutum* between measured and calculated ε_p. One should keep in mind that the isotope fractionation model can only serve as an approximation of ε_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 ε_p is an accurate estimate of C_i fluxes. As discussed above, both an underestimate of CO_2 efflux and the neglect of HCO_3^- efflux may strongly influence flux estimates. In analogy to our calculation of C_i fluxes, HCO_3^- efflux is considered negligible in the isotope fractionation model. Therefore, it is difficult to assess how additional HCO_3^- efflux might affect ε_p. An increase in the relative contribution of HCO_3^- uptake to total uptake causes a decrease in ε_p that would make the discrepancy between calculated and measured fractionation even larger. On the other hand, higher ε_p values could theoretically be achieved if massive C_i efflux occurred at the same time. Much of the discrepancy between measurement and model calculations could also be explained in the absence of HCO_3^- efflux: higher rates of CO_2 efflux would lead to higher $CO_2:HCO_3^-$ 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_2 efflux by a factor of two would cause an increase in ε_p by ~5‰.

These considerations indicate that a systematic underestimate in CO_2 efflux may, in part, be responsible for the low ε_p values calculated by the fractionation model. Nevertheless, we were able to produce realistic trends in the ε_p versus $[CO_{2,aq}]$ relationship on the basis of our C_i flux data, which provides evidence that the frequently observed decrease in ε_p in response to diminishing CO_2 supply may be the combined result of the gradual induction of HCO_3^- uptake and a decrease in CO_2 leakage.

In conclusion, results from this study indicate that, in spite of species-specific differences in certain aspects of C_i acquisition, the overall responses of the two marine diatoms tested here to changes in CO_2 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_2 and HCO_3^- demonstrate the capacity of these organisms to maintain high photosynthetic rates in response to large variations in the marine carbonate system. Even at CO_2 concentrations exceeding current levels in ocean surface waters by a factor of five, CO_2 uptake is supplemented by direct uptake of HCO_3^-. On the other hand, our results clearly indicate that CO_2 remains an important substrate for photosynthesis even at CO_2 concentrations well below CO_2 levels typically encountered in the ocean. Under the assumption that CO_2 uptake requires less energy than HCO_3^- uptake, the advantage of a highly efficient CO_2 uptake mechanism may be to facilitate photosynthetic performance in a light-limited environment.

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