

The role of extracellular carbonic anhydrase activity in inorganic carbon utilization of *Phaeocystis globosa* (Prymnesiophyceae): A comparison with other marine algae using the isotopic disequilibrium technique

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Abstract

The utilization of inorganic carbon species by the marine microalga *Phaeocystis globosa* (Prymnesiophyceae) and several other algal species from different taxa, was investigated by determining the time course of ¹⁴C incorporation in isotopic disequilibrium experiments. From these kinetic data, conclusions can be drawn about the carbon species, CO₂ or HCO₃⁻, that is being utilized. By comparing the uptake kinetics in the absence and presence of acetazolamide (AZ) or dextran-bound sulfonamide, inhibitors of external carbonic anhydrase (CA), it was determined that *P. globosa*, *Dunaliella tertiolecta*, and some strains of *Emiliana huxleyi* do use HCO₃⁻ by extracellular, CA-catalyzed conversion to CO₂, which then diffuses across the membrane. *Nannochloropsis*, *Thalassiosira pseudonanna*, and often *Synechococcus* use HCO₃⁻ without extracellular conversion. *Thalassiosira punctigera*, some strains of *E. huxleyi*, and *Rhodomonas* sp. use exclusively free CO₂. The presence of extracellular CA activity in *Phaeocystis* is not constitutive but is induced under low inorganic carbon conditions. Thus, marine microalgae show variability in carbon acquisition strategy for one single species, depending on external conditions, and in carbon acquisition strategy between species. Determining AZ-induced changes in carbon uptake kinetics provides a sensitive test for the presence of extracellular CA activity. With the potentiometric method, no CA activity could be measured, whereas with the isotopic disequilibrium technique, significant CA activity could be detected.

For photosynthesizing aquatic macro- and microphytes, the chemical composition of the unstirred layer surrounding the cells differs from that of the bulk of the medium. Uptake of CO₂ or any other nutrient by the cell depletes the immediate environment and creates a concentration gradient. In a steady state, the uptake of CO₂ is balanced by diffusion from the bulk medium into the unstirred layer and by production of CO₂ from HCO₃⁻ and CO₃²⁻ in the unstirred layer itself (Wolf-Gladrow and Riebesell 1997). Because of the slow rate of formation from HCO₃⁻ and diffusional limitation through the unstirred layer around the cells, the availability of CO₂ may limit photosynthesis and growth of marine algal species (Riebesell et al. 1993; Chen and Durbin 1994). Strategies used by aquatic species to overcome these limitations include the active uptake of one of the carbon species across one of the membranes, either the plasmamembrane or the chloroplast envelope that separate the external medium from the site of fixation. This mechanism is commonly referred to as carbon concentrating mechanism (CCM). Another strategy is the carbonic anhydrase-catalyzed extracellular conversion of HCO₃⁻ to CO₂, followed by diffusion of CO₂ across the membrane. These mechanisms are often only induced by conditions where CO₂ is

limiting (Badger and Price 1992). In cyanophytes and fresh water microalgae, both the high rates of carbon fixation (sustainable only by actively transporting inorganic carbon) and the high intracellular inorganic carbon concentration under low CO₂ conditions indicate the presence of a carbon concentrating mechanism. In marine microalgae, the situation seems to be more diverse: in some macrophytes and in some microalgae, the rate of diffusion of CO₂ appears to be sufficient to sustain the measured photosynthetic carbon fixation. In others, a carbon-concentrating mechanism is assumed to be present (Falkowski and Raven 1997).

The isotopic disequilibrium technique used in this study makes use of the relatively slow equilibration between CO₂ and HCO₃⁻. The addition of trace amounts of ¹⁴CO₂ to a solution at a constant, higher pH will generate a transient isotopic disequilibrium as the majority of the ¹⁴C is initially present as ¹⁴CO₂, whereas the unlabeled inorganic carbon is distributed over CO₂, HCO₃⁻, and CO₃²⁻ at equilibrium values (Lehman 1978; Findenegg 1980; Miller 1985; Espie and Colman 1986; Espie et al. 1986; Staal et al. 1989; Eighmy et al. 1991). The time course for equilibration can be calculated, given the appropriate rate constants for the experimental conditions (pH, T, salinity). Incorporation of label in photosynthetic products by cells that are fixing carbon at steady state will reflect the change in specific activity of the carbon species (CO₂, HCO₃⁻, or a mix of both) taken up from the medium. We make use of the difference in ¹⁴C incorporation kinetics between control and acetazolamide (AZ)-treated cell suspension to determine the presence of extracellular carbonic anhydrase (CA). (AZ is allegedly an inhibitor of extracellular, but not intracellular, carbonic an-

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hydrase activity; see Axelsson et al. 1995.) Regardless of the labeled carbon species added, cells with extracellular CA activity show a linear incorporation of label, whereas the AZ-treated cells show the kinetics of the carbon species actually taken up (Miyachi et al. 1983; Tsuzuki 1983; Williams and Turpin 1987).

Current understanding of marine primary production is not sufficient to faithfully model the role of the biological carbon pump in the oceanic uptake of anthropogenic carbon dioxide. One of the largest uncertainties in models for oceanic carbon dioxide exchange is the level of response of the photosynthetic organisms responsible for the carbon precipitation in the form of organic material (Sarmiento and Le Quere 1996). This response will depend on whether the species that are dominating the oceanic primary production are CO₂ users and are thus possibly carbon limited (Riebesell et al. 1993), or do possess a CCM and are thus capable of using HCO₃⁻ and are insensitive to changes in the carbon concentration in the environment (Tortell et al. 1997). *Phaeocystis* was chosen in this study, as it is a ubiquitous colony-forming species frequently dominating the phytoplankton during the productive period. A contribution of 40–90% to total phytoplankton numerical abundance has often been reported (reviewed by Davidson and Marchant 1992). During the 1998 spring bloom in Dutch coastal waters, *Phaeocystis* contributed 90–97% of total phytoplankton abundance over a 4-wk period (pers. observ.). For *Phaeocystis*, there was no conclusive evidence for the mechanism of carbon uptake.

Materials and methods

Algal strains and culturing conditions—*P. globosa* (Prymnesiophyceae), *Synechococcus* sp. WH7803 (Cyanophyta), *Thalassiosira punctigera* (Bacillariophyceae), *Thalassiosira pseudonanna* (Bacillariophyceae), *Dunaliella tertiolecta* (Chlorophyta), and *Rhodomonas* sp. (Cryptophyta) were all from our own collection. The highly calcifying strain of *Emiliania huxleyi* (Prymnesiophyceae) was a kind gift from Dr. Ulf Riebesell (Alfred Wegener Institut, Bremerhaven, Germany). The other strains were from our own collection. *Nannochloropsis* sp. (Eustigmatophyta) was a kind gift from Dr. Assaf Sekenik (National Institute of Oceanography, Haifa, Israel).

Cells were grown in medium based on filtered, sterilized (by autoclaving) natural seawater supplemented with minor salts and trace elements as described by Veldhuis and Admiraal (1987) and, unless stated otherwise, supplemented with 2.4 mM bicarbonate. Nitrate, phosphate, and, in the case of diatom cultures, silicate were added in concentrations of 340, 18, and 50 μM, respectively. Cultures were grown in 1-liter serum bottles, placed on a rolling device, and incubated at 12°C and a photon flux density of 150 μmol m⁻² s⁻¹ provided by Philips TL40W/33 fluorescent tubes in a 14 h light: 10 h dark cycle.

Isotopic disequilibrium technique—*Phaeocystis* cells were harvested on day 7 after inoculation or at the times indicated; other cell species were at late log phase. Cells were harvested by centrifuging the cells at 100 × g for 10 min in a swing-out rotor. The pellet was resuspended in fresh natural

seawater (NSW). The pH of the suspension was determined to two decimal places, and if the value deviated more than 0.3 pH units from pH 8.5, the suspension was diluted five-fold with fresh NSW, centrifuged, and resuspended again. The resulting loose pellet was resuspended in NSW to a density varying between 1.1 × 10⁶ to 12.1 × 10⁶ cells ml⁻¹. Cell numbers of samples taken from the culture and the experimental cell suspension were determined with a Buerger hemocytometer after they were fixed with acid Lugol (10 μl ml⁻¹ cell suspension). In order to reach steady-state photosynthesis, the cell suspension was preilluminated for 5 min with a photon flux density of 150 μmol m⁻² s⁻¹ provided by a 24-V slide projection lamp type 7748S (Philips, Eindhoven, The Netherlands). The light intensity was measured with a SKP 200 quantum meter (Skye Instruments, Powys, UK) at the level of the cell suspension. The temperature of the cell suspension was maintained at 15°C by the water mantle surrounding the cuvette holding the cell suspension. When inhibitors of carbonic anhydrase or anion transport were used, they were included 15 min before the start of the preillumination.

The experiment was started by adding 49 μl of ¹⁴C-labeled NaHCO₃ (resulting in a specific activity of 80 kBq μmol⁻¹) in 50 mM bis-Tris-propane-HCl pH 7.0, to 4 ml of the illuminated cell suspension (addition of the label did not alter the pH of the cell suspension) and swirling the suspension for about 3 s. By first bringing the ¹⁴C-labeled NaHCO₃ to pH 7.0, about 7.5% of the label is in the CO₂ form, whereas after establishment of chemical equilibrium in the experimental solution with a pH of 8.5, this would be 0.2%. From the cell suspension, 200-μl samples were taken in short (3–5 s intervals). Carbon-fixing activity in these samples was stopped by dilution in 1 ml 80% acetone. To remove unfixed ¹⁴C, 25-μl 8 N HCl was added to the samples and an air stream forced over the samples for 2 h. To avoid quenching of the scintillation fluid, the samples were neutralized by 50-μl 4 N KOH, and the acetone was removed by keeping the samples at 65°C for 50 min in a water bath. To the samples, 4 ml of scintillation fluid (Ultima Gold, Packard Instrument, Meriden, Connecticut) was added, and fixed radioactivity was assayed. The large volume of scintillation fluid relative to the sample volume ensured that the counting efficiency was more than 90% and did not vary among samples.

Nonlinear fitting procedure—The isotopic disequilibrium method makes use of the relatively slow equilibration between CO₂ and HCO₃⁻. A mathematical description of the time course of incorporation of radioactive carbon was derived from the equations quantifying the contribution of both CO₂ and HCO₃⁻ (Findenegg 1980; Miller 1985; Espie and Colman 1986; Espie et al. 1986). The specific activity of CO₂ and HCO₃⁻ changes with time according to

$$SA_{CO_2,t} = SA_{DIC} + \Delta SA_{CO_2} e^{-\alpha t} \quad (1)$$

$$SA_{HCO_3,t} = SA_{DIC} + \Delta SA_{HCO_3} e^{-\alpha t} \quad (2)$$

The rate of incorporation of radioactivity is given by

$$d(DPM)_t/dt = V_{CO_2} SA_{CO_2,t} + V_{HCO_3} SA_{HCO_3,t} \quad (3)$$

Integration and the condition that at $t = 0$ no radioactivity has been incorporated yet yields

$$\begin{aligned} \text{DPM}_t = & V'_{\text{CO}_2}(\alpha_1 t + (\Delta\text{SA}_{\text{CO}_2}/\text{SA}_{\text{DIC}}) \cdot (1 - e^{-\alpha_1 t}))/\alpha_1 + \dots \\ & + V'_{\text{HCO}_3^-}(\alpha_2 t + (\Delta\text{SA}_{\text{HCO}_3^-}/\text{SA}_{\text{DIC}}) \cdot (1 - e^{-\alpha_2 t}))/\alpha_2 \\ (V'_{\text{CO}_2} = & V_{\text{CO}_2} \cdot \text{SA}_{\text{DIC}} \text{ and } V'_{\text{HCO}_3^-} = V_{\text{HCO}_3^-} \cdot \text{SA}_{\text{DIC}}), \end{aligned} \quad (4)$$

where DPM_t is the fixed radioactivity at time t (in disintegrations min^{-1} ; dpm); SA_{CO_2} , and $\text{SA}_{\text{HCO}_3^-}$ are the specific activity at time t of CO_2 and HCO_3^- , respectively; SA_{DIC} is the specific activity of total dissolved inorganic carbon and thus of all the individual carbon species at equilibrium (in dpm mol^{-1}); and $\Delta\text{SA}_{\text{CO}_2}$ and $\Delta\text{SA}_{\text{HCO}_3^-}$ are the changes in specific activity from $t = 0$ to $t = \infty$ of CO_2 and HCO_3^- , respectively (in dpm mol^{-1}). In a typical experiment where the pH of the solution with ^{14}C label is 7.0 and the pH of the cell suspension is 8.5, $\Delta\text{SA}_{\text{CO}_2}/\text{SA}_{\text{DIC}}$ is 33.01, and $\text{SA}_{\text{HCO}_3^-}/\text{SA}_{\text{DIC}}$ is 0.08; V_{CO_2} and $V_{\text{HCO}_3^-}$ are the rate of fixation of carbon in acid stable compounds originating in uptake from the bulk solution of CO_2 and HCO_3^- , respectively (in mol s^{-1} ; note that V'_{CO_2} and $V'_{\text{HCO}_3^-}$ are in dpm s^{-1}); α_1 and α_2 , are salinity-, temperature- and pH-dependent rate constants for the equilibration of the specific activity of CO_2 and HCO_3^- , respectively, calculated as in Espie and Colman (1986) in seconds, in a typical experiment where the pH of the solution with ^{14}C label is 7.0 and the pH of the cell suspension is 8.5, α_1 is 0.0729, and α_2 is 0.0832.

Using the nonlinear fitting function of Prism software version 2.01 (Graphpad, San Diego, California), Eq. 4 was fitted to the data points with V_{CO_2} and $V_{\text{HCO}_3^-}$ as free-running parameters. The same software package was used to compare fits to the data of two different equations (Motulski 1999).

Carbonic anhydrase activity assay—Carbonic anhydrase activity was determined essentially according to Findenegg (1979). *Phaeocystis* cells were concentrated by centrifuging for 5 min at $100 \times g$ and resuspending the loose pellet to a density of about 1.4×10^6 cells ml^{-1} in 10 ml of filtered NSW buffered at pH 8.3 by 20 mM Hepes and kept at a temperature of 0°C . Ten milliliters of seawater, equilibrated with 100 kPa of pure CO_2 in the gas phase, was added, and the resulting acidification was traced on a chart recorder. Seawater without cells and cell suspensions to which 20 μM acetazolamide (an inhibitor of periplasmic carbonic anhydrase) was added were used as controls. The effectiveness of the inhibition by AZ was tested on 2,200 ng ml^{-1} bovine carbonic anhydrase dissolved in NSW. Enzyme activity was determined as enzyme units (EU); 1 EU = $(T_u/T_c) - 1$, where T_u and T_c are the times needed for a pH decrease from 8.1 to 6.5 in the controls (uncatalyzed) and the uninhibited cell suspensions (catalyzed), respectively.

Chemicals—Dextran-bound sulfonamide (MW dextran 6000) was obtained from Synthelec AG (Lund, Sweden), acetazolamide (*N*-[5-sulfamoyl-1,3,4-thiadiazol-2-yl]-acetamide) and carbonic anhydrase from bovine erythrocytes from Sigma (Zwijndrecht, The Netherlands), and ^{14}C -labeled

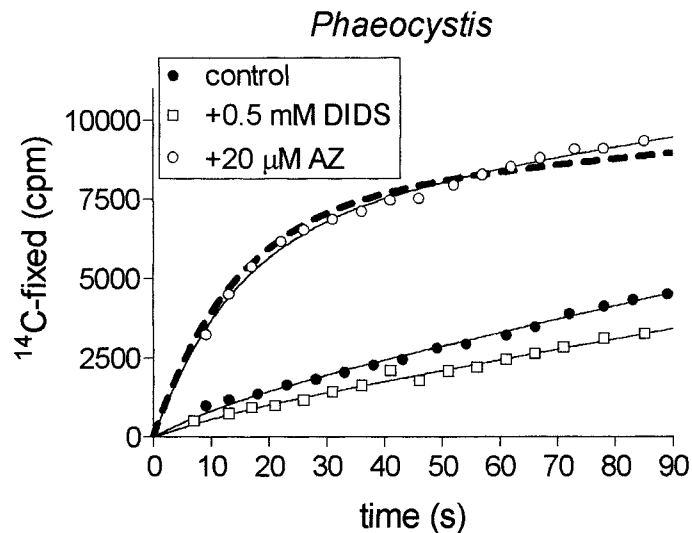


Fig. 1. The effect of 20 μM AZ and 500 μM 4'-diisothiocyanatostilbene-2,2-disulfonic acid on ^{14}C incorporation in *Phaeocystis* cells from the late log phase. The thin continuous lines fitted to the data are based on the rate of equilibration and the relative incorporation of ^{14}C from CO_2 and that from HCO_3^- . The broken line indicates the best fit of the equation assuming only use of CO_2 and no contribution of HCO_3^- . The ^{14}C label buffered at pH 7 was added to a cell suspension at pH 8.48.

NaHCO_3 from Amersham Life Science (Little Chalfont, UK).

Results

Effect of AZ on kinetics of ^{14}C incorporation—The isotopic disequilibrium technique has been used primarily to determine which inorganic carbon species (CO_2 or HCO_3^-) is transported across the membrane. In the traditional isotopic disequilibrium experiments, one of the prerequisites for application is the absence of extracellular carbonic anhydrase activity, as this enzyme rapidly dissipates the isotopic disequilibrium and will show a linear incorporation of ^{14}C regardless of which C_i species is used (Espie et al. 1984; Miller 1985). In this study, we used the change in kinetics of ^{14}C incorporation caused by the inhibition of carbonic anhydrase activity by acetazolamide (AZ) as a pseudoquantitative measure for extracellular carbonic anhydrase activity.

In Fig. 1, the effect of 20 μM AZ on the time course of ^{14}C incorporation in *Phaeocystis* cells from the late log phase is shown. The lines fitted to the data are based on rate of equilibration (calculated from rate constants for carbon species interconversion appropriate for the experimental pH, salinity, and temperature) and the relative incorporation of ^{14}C from CO_2 and from HCO_3^- (Eq. 4). In the control experiments, the data could only be fitted satisfactorily by an equation that incorporated contributions of both CO_2 and HCO_3^- (Eq. 4). In contrast, when the cells were incubated in a medium containing 20 μM of AZ, fitting an equation to the data that describes CO_2 incorporation only (Eq. 4, $V'_{\text{HCO}_3^-} = 0$) yielded a line that was in most cases indistinguishable ($P < 0.05$) from the one that resulted from the equation with

both HCO_3^- and CO_2 contributions. This indicates that the contribution of HCO_3^- in photosynthetic carbon uptake in AZ-treated cells is insignificant. The apparent contribution of both C_i species was not affected by the inclusion of 4'4'-diisothiocyantostilbene-2,2-disulfonic acid, an inhibitor of anion exchange and of anion channels.

Effect of membrane impermeable inhibitors of carbonic anhydrase—To ascertain that AZ inhibits extracellular carbonic anhydrase activity only, its effects were compared with those of dextran-bound sulfonamide (DBS) that, because of its large molecular weight, is unquestionably membrane impermeable. The effect of AZ and DBS on the kinetics of ^{14}C incorporation were almost identical when concentrations were used that showed equivalent inhibition of bovine carbonic anhydrase activity in a potentiometric assay (data not shown). This is further confirmation that the effect on ^{14}C incorporation of AZ is indeed due to inhibition of extracellular CA activity.

Induction of extracellular carbonic anhydrase in Phaeocystis—Since the effect of carbonic anhydrase in increasing the flux of CO_2 for photosynthesis is only relevant when the diffusive resistance of the boundary layer for CO_2 is a limiting factor in photosynthesis, it could be expected that the activity of carbonic anhydrase is induced under low- CO_2 conditions. When grown in batch, the cell culture will gradually increase the pH and reduce total inorganic carbon, thus creating CO_2 limitation. From the development of cell density, culture pH, and carbonic anhydrase activity in batch cultures of *Phaeocystis*, it is obvious that with decreasing CO_2 availability, the activity of carbonic anhydrase increases (Fig. 2). As this induction could be the result of either an increase in pH or a decrease in carbon availability, the CA activity was determined in a cell culture in which the pH was kept constant at 8.0 by adding 5 mM Tricine and the initial inorganic carbon concentration was lowered to 0.5 mM. At the end of the log phase, the pH was still 8.0, but carbonic anhydrase activity was nevertheless induced (Fig. 3). This result suggests that carbonic anhydrase induction is a function of the carbon availability and not of medium pH.

Estimating the carbonic anhydrase activity in the boundary layer—Since we established that the apparent utilization of HCO_3^- is due to CA catalyzed conversion of HCO_3^- and CO_2 , we analyzed the data accordingly. The catalysis of the equilibration between CO_2 and HCO_3^- by carbonic anhy-

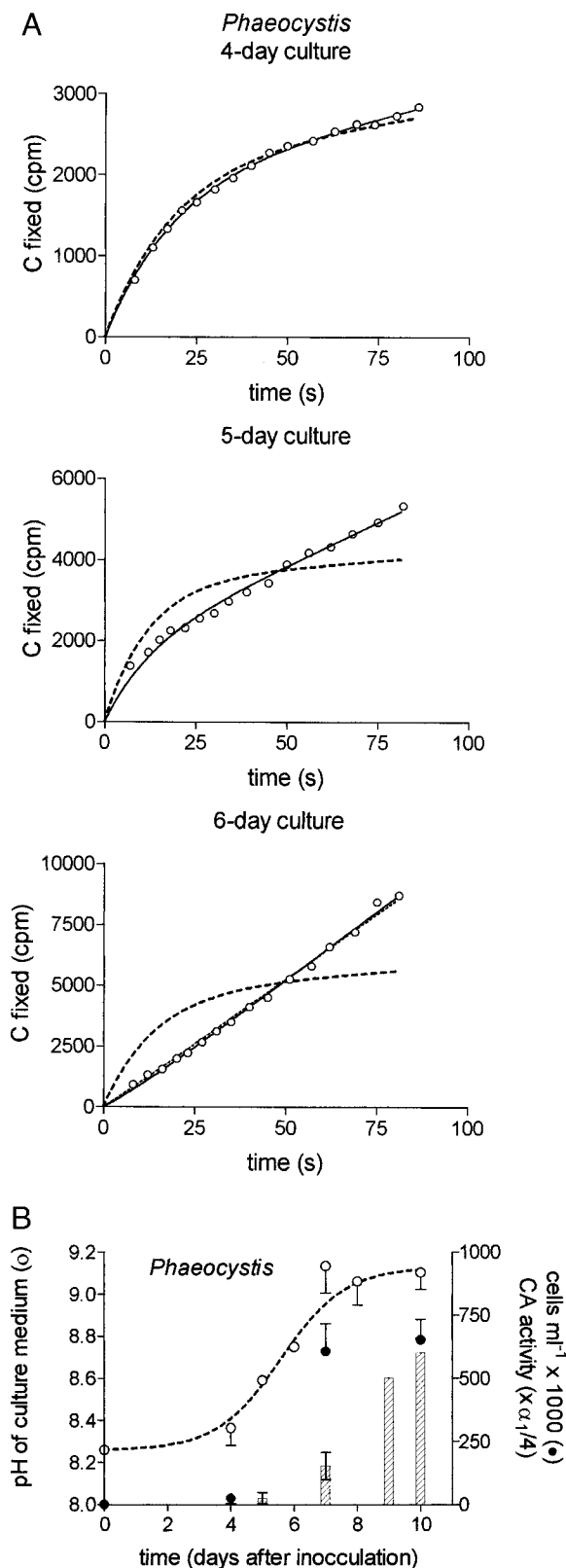


Fig. 2. Effect of age of *Phaeocystis* batch culture on carbonic anhydrase activity. A. Kinetics of ^{14}C incorporation by *Phaeocystis* cells from batch cultures on different days after inoculation. The continuous line gives the best fit of the equation describing utilization of both HCO_3^- and CO_2 . The broken line gives the best theoretical ^{14}C incorporation curve assuming that only CO_2 is utilized. B. Cell density, culture pH, and carbonic anhydrase activity (bars) in batch cultures of *Phaeocystis*. Cell density and culture pH were determined at the times indicated after inoculation. The bars in the graph indicate the carbonic anhydrase activity, as determined

from experiments as in Fig. 2A and expressed as the increase in the rate constants (α_1 and α_2) of Eqs. 1, 2, and 4 as described in Fig. 4 (the values can be obtained by taking the values of the right y-axis and dividing them by 4). The dotted line is a fit of the equation for logistic growth to the pH of the culture.

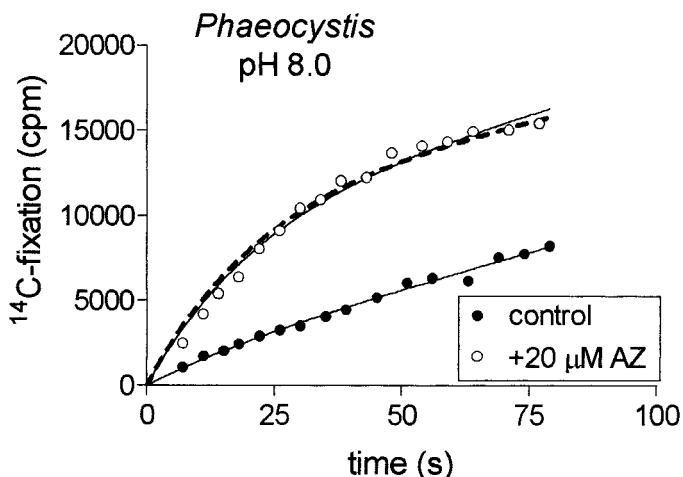


Fig. 3. Effect of 20 μM acetazolamide on the kinetics of ^{14}C incorporation by *Phaeocystis* cells from a batch culture in which the pH was buffered at 8.0 by 5 mM Tricine and the inorganic carbon was reduced to 0.5 mM (see Fig. 1 for further details).

drase can be modeled by increasing the rate constant α_1 in Eq. 4. Modeling this increase in rate constant leads to the curves shown in Fig. 4, where it is assumed that the only C_i species crossing the membrane is CO_2 . When these curves are fitted to the data of Fig. 1, the increase in α_1 needs to be 38 ± 13 times, equivalent to 37 EU of CA activity.

Although the isotopic disequilibrium experiments indicate the presence of extracellular CA, no CA activity could be detected (data not shown) with the potentiometric method, which is based on the rate of pH change induced by adding an aliquot of CO_2 -saturated solution to the enzyme-containing solution. This apparent contradictory result can be resolved by assuming that the extracellular carbonic anhydrase activity is located in the periplasmic space inside the boundary layer and not homogeneously distributed in the bulk solution. Such a localization is consistent with both the results of the isotopic disequilibrium experiments and with the failure to detect CA activity potentiometrically. (For a cell with a diameter of 5 μm and a cell density of 1.4×10^6 cells ml^{-1} , the effective boundary layer is only 0.022% of the total cell suspension volume. The periplasmic CA activity of 38 EU would lead to an apparent CA activity in the bulk phase of less than 0.01 EU. This explains why no CA activity could be detected in late log phase *Phaeocystis* cultures with the potentiometric method, as 0.01 EU is well below the detection limit of this method.)

Effect of inhibition of CA activity on steady-state ^{14}C fixation—Although in theory the presence of CA would enhance the CO_2 delivery extracellularly at the plasmamembrane, and it is found that CA activity is induced under conditions of low carbon supply, one would expect that inhibition of CA would inhibit the steady state photosynthetic rate. To test this assumption, ^{14}C incorporation after 12 min of steady-state illumination in cells in the presence and absence of 20 μM AZ was compared (Fig. 5). As expected, AZ seems to have maximal effect at the intermediate values of pH; at the lower pH values, the diffusional flux of CO_2

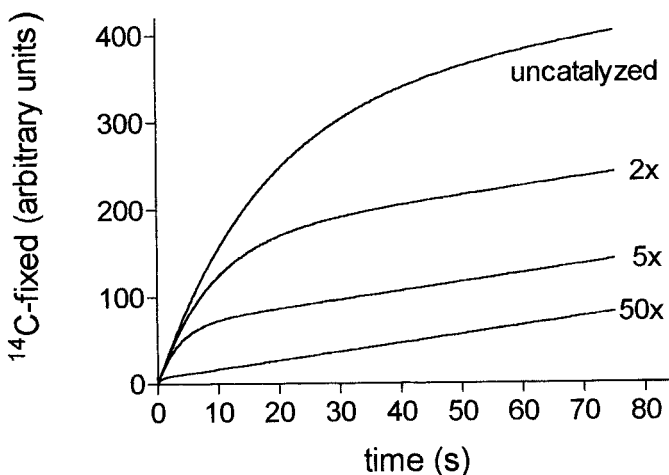


Fig. 4. Effect of changes in the rate constant of $^{14}\text{CO}_2$ equilibration. Theoretical curves are for ^{14}C incorporation, assuming that the only C_i species crossing the membrane is CO_2 . The carbonic anhydrase catalyzed equilibration between CO_2 and HCO_3^- is modeled by increasing the rate constants α_1 and α_2 (modified after Espie and Calvin 1987).

is sufficient and the conversion of HCO_3^- to CO_2 insignificant, while at the higher pH, the photosynthetic rate is determined by the low equilibrium concentration of CO_2 and not by the flux of CO_2 to the plasmamembrane.

Presence of extracellular carbonic anhydrase in other marine algal species—To test its validity, the isotopic disequilibrium procedure was used to determine the carbon species taken up by a known HCO_3^- user. Several lines of evidence indicate that the C_i species crossing the membrane in the eustigmatophyte *Nannochloropsis* is HCO_3^- (Merrett et al. 1996; Sukenik et al. 1997). This species does not have extracellular CA (Sukenik et al. 1997). *Nannochloropsis* therefore provided a test for the validity of the use of the isotopic disequilibrium technique to discriminate between direct and indirect (through CA catalyzed conversion to CO_2) utiliza-

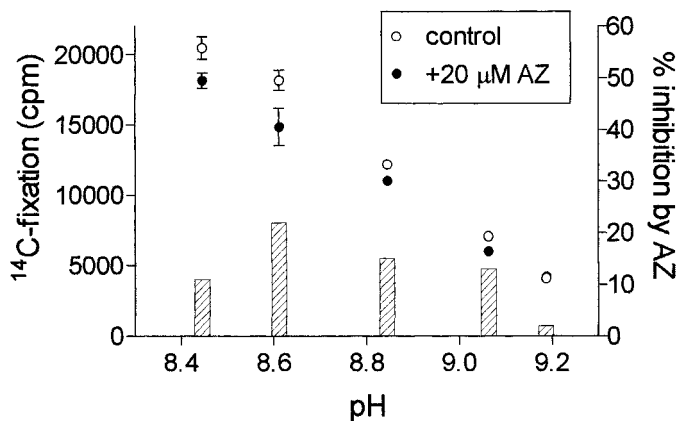


Fig. 5. The effect of 20 μM acetazolamide on the ^{14}C incorporation during 12 min in *Phaeocystis* cells constantly illuminated. The shaded bars indicate the percentage of the reduction of the steady-state carbon incorporation by acetazolamide.

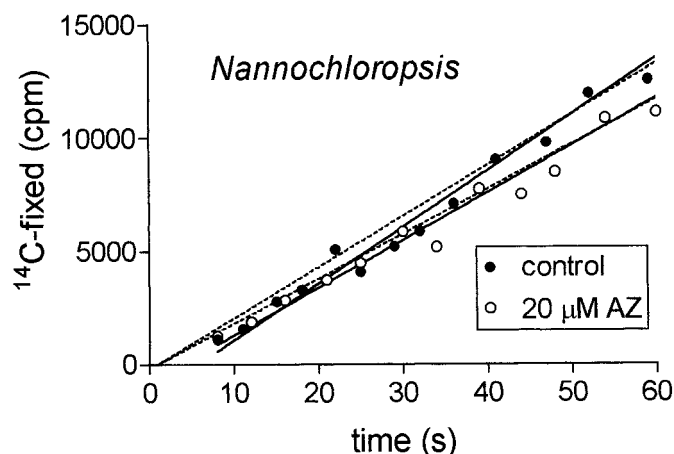


Fig. 6. ^{14}C incorporation in *Nannochloropsis* sp. in the presence and absence of $20\ \mu\text{M}$ acetazolamide. The continuous line gives the best fit of the equation describing utilization of both HCO_3^- and CO_2 . The broken line gives the best theoretical ^{14}C incorporation curve, assuming that only HCO_3^- is utilized (see Fig. 1 for further details).

tion of HCO_3^- . The kinetics of ^{14}C incorporation in *Nannochloropsis* cells closely match the theoretical curve for a HCO_3^- -only user (Fig. 6). Including $20\ \mu\text{M}$ AZ did not influence the time course of ^{14}C incorporation, indicating that extracellular CA activity does not play a role in the HCO_3^- utilization.

In Table 1, the results obtained with several other species are compiled, showing large differences between species regarding the pH that can be reached in the late log phase of a batch culture. Furthermore, the data show the variability in the contribution of CA catalyzed conversion of HCO_3^- to CO_2 between species and the variability between different strains of one species (*E. huxleyi*). In addition, the different ability of species within one genus (*Thalassiosira*) to either take up HCO_3^- (*T. pseudonanna*) or CO_2 (*T. punctigera*) is apparent. Of the species and strains tested, three (*Nannochloropsis*, *Synechococcus*, and *T. pseudonanna*) apparently make use of HCO_3^- by transporting it across the plasma-membrane. In these three species, the pH of the medium at the late log stage was highest and the addition of AZ had no significant effect on the kinetics of ^{14}C incorporation. In all other species tested, the C_i species crossing the membrane (tested in the presence of AZ) was CO_2 . CO_2 use was accompanied by the ability of induction of extracellular CA activity in *Phaeocystis*, a strain of *E. huxleyi*, and *Dunaliella*.

Discussion

In this study, the isotopic disequilibrium technique, or "kinetic reaction approach" (Miller 1985), was used to monitor the change in extracellular CA activity in *Phaeocystis* cultures. The kinetic reaction approach is based on a comparison among the theoretical equilibration kinetics and the measured kinetics of ^{14}C fixation. This comparison yields a relative value for the contributions of HCO_3^- and CO_2 in the carbon that is photosynthetically fixed. By performing the experiment in both the presence and absence

Table 1. Culture end pH, carbon species used, and external carbonic anhydrase activity of algal species studied.*

Species	Culture pH	C_i species	External CA
<i>Phaeocystis globosa</i>	9.14	CO_2	+(38.2 + 13.6)
<i>Emiliania huxleyi</i> (light coccolith former)	9.29	CO_2	+(17.0 + 0.7)
<i>E. huxleyi</i> (heavy coccolith former)	8.80	CO_2	—
<i>Dunaliella</i>	8.69	CO_2	+(20.0 + 1.7)
<i>Thalassiosira punctigera</i>	8.90	CO_2	—
<i>T. pseudonanna</i>	9.77	HCO_3^-	—
<i>Nannochloropsis</i>	10.08	HCO_3^-	—
<i>Synechococcus</i>	9.40	HCO_3^-	—

* The column labeled "culture pH" gives the pH value of the medium at the late log phase of the batch culture. The column " C_i species" gives the carbon species actually crossing the membrane based on the kinetics of ^{14}C incorporation under conditions where extracellular CA activity is inhibited (in the presence of $20\ \mu\text{M}$ AZ). The column "external cA" indicates whether inclusion of $20\ \mu\text{M}$ AZ resulted in a significant change (+) or not (—) in the kinetics of the ^{14}C incorporation. Typically, in cell suspensions with extracellular CA activity, adding AZ to the medium resulted in shift of apparent HCO_3^- uptake kinetics to CO_2 uptake kinetics (see Fig. 1). The figure in brackets represents the increase of α_1 due to carbonic anhydrase activity in cultures, 7 d after subculturing, calculated by fitting Eq. 4 to the data obtained in the absence of AZ, assuming uptake of CO_2 only.

of AZ, it can be determined whether the HCO_3^- contribution is due to transport of the HCO_3^- ion across the membrane or is due to CA-catalyzed equilibration yielding higher CO_2 concentrations at the plasma membrane (resulting from additional diffusion of HCO_3^- from the bulk solution across the unstirred layer). Tsuzuki et al. (1983), Miyachi et al. (1983), and Williams and Turpin (1987) have used the effect of AZ in the isotopic disequilibrium technique to determine the carbon species taken up in *Chlorella* and *Chlamydomonas*.

Although it was not the objective of this study to establish the sensitivities of the carbonic anhydrase assay methods, the kinetic reaction approach proved to be much more sensitive than the traditional potentiometric method. With the latter, we could not measure any extracellular CA in *Phaeocystis* cultures, and with the isotopic disequilibrium technique, it was observed that late log-phase cultures possessed sufficient CA to completely abolish the disequilibrium effect. This discrepancy is caused by the localization of the extracellular CA in the periplasmic space. The exchange of solutes between the bulk solution and the periplasmic space, by definition located within the unstirred layer surrounding the cell, occurs by diffusion only. The CA concentration within the small volume of the unstirred layer is apparently high enough to speed up the equilibration of inorganic carbon species such that disequilibrium effects are reduced. But this local, high activity is not enough to induce an increase of the hydration rate of CO_2 in the bulk phase. This apparent discrepancy has also been observed in photosynthetic characteristics of macroalgae (Mercado et al. 1997a). The presence of extracellular CA activity, not detectable by conventional means, was deduced from the different kinetics of oxygen evolution after adding CO_2 to photosynthesizing

Porphyra tissue. Low levels of extracellular CA have also been inferred from experiments where the photosynthetic rates at high pH were reduced by the membrane-impermeable, CA-specific inhibitors AZ and dextran-bound sulfonamides (Axelsson et al. 1995).

One experimental difficulty in the interpretation of the results is the need to concentrate the cells by centrifugation, possibly leading to cell damage and release in the medium of intracellular carbonic anhydrase. Release of carbonic anhydrase into the medium would lead to a dissipation of the disequilibrium throughout the medium, not just in the periplasmic space. The observation that concentrated cell suspensions do not show any increase in the rate of equilibration as measured with the traditional potentiometric method normally used to determine CA activity rules out this possibility. A second indication that release of intracellular CA could not cause the apparent HCO_3^- utilization is that dilution of the cell suspension does not influence the kinetics of ^{14}C incorporation (data not shown).

In recent years, the question whether external carbonic anhydrase catalyzed conversion of HCO_3^- to CO_2 is a requirement for the photosynthetic rates observed in algae has been addressed for a number of species (see review by Sultemeyer et al. 1993). In the freshwater species *Chlamydomonas* and *Chlorella*, abundant extracellular CA activity can be determined with the potentiometric method, and the CA activities increase dramatically when cells are transferred from a high- C_i to a low- C_i medium (for references, see Sultemeyer et al. 1993). Compared with the situation in freshwater algae, the situation in marine algae is more complicated. The level of CA activity is sometimes so low it cannot be measured by the potentiometric method (Axelsson et al. 1995; Mercado et al. 1997a; this study). And besides the plasticity of extracellular CA activity, induction in low CO_2 or carbon limitation of *Porphyra leucosticta* (Mercado et al. 1997b), diatoms (Nimer et al. 1997), and *Phaeodactylum tricorutum* (John-McKay and Colman 1997), there is also variability between strains such as *Skeletonema* (Korb et al. 1997; Nimer et al. 1997) and *Phaeodactylum tricorutum* (John-McKay and Colman 1997).

Seemingly, our results with the non-coccolith-forming and the heavily coccolith-forming strains of *E. huxleyi* appear to differ from earlier derived models (Dong et al. 1993). In these models, the non-coccolith-forming strain obtains inorganic carbon by diffusive entry of free CO_2 , whereas the coccolith-forming strain actively takes up HCO_3^- and uses this in the formation of coccoliths (by precipitation of calcite CaCO_3) under simultaneous release of CO_2 that can be used for photosynthetic fixation. However, the relevant period in an isotopic disequilibrium experiment only lasts about 15 to 20 s. Therefore, the contribution of ^{14}C that has entered in the form of HCO_3^- , transported into the calcisomes, subsequently released from the calcisome in the form of CO_2 and then photosynthetically fixed, might be substantial after a longer period of incubation. In the short time the experiment takes, the acid stable carbon products therefore will be dominated by the CO_2 that has diffusively entered the cells directly. Indeed, the absence of extracellular CA activity in the coccolith-forming strain is in agreement with the use of HCO_3^- , entering the cell via a transporter, as the dominant

inorganic carbon source, or in agreement with low carbon demand.

The need for catalyzed HCO_3^- - CO_2 equilibration at high pH—It is assumed that in marine phytoplankton, carbon limitation of photosynthesis and growth does not occur under normal conditions. Although arguments that diatoms are actually C_i limited in growth have been presented (Riebesell et al. 1993), other data suggest that in diatoms, a carbon concentrating mechanism will alleviate a possible limitation based on CO_2 diffusion alone. That marine algae can encounter low CO_2 supply situations is evident from field measurements where undersaturation of CO_2 as low as 120 ppm (Codispoti et al. 1982) or 90 ppm (pers. observ.) were recorded, correlating with a pH of about 8.6. During a *Phaeocystis* bloom in the North Sea, an increase of the pH from 7.9 to 8.7 was recorded (Brussaard et al. 1996). Hinga (1992) reports the collection of *Phaeocystis* from water with a pH ranging from 8.8 to 9.1. In water where the pH of the bulk is 8.6, the pH inside a *Phaeocystis* colony can increase to 9.1 (Lubbers et al. 1990). In our experimental system, the low CO_2 concentration present at all these pH values induce carbonic anhydrase activity (Fig. 2B). In the present study, we found that in a batch culture, growth of *Phaeocystis* can increase the pH to 9.2. Although the presence of CA activity can increase the rate of CO_2 supply at the plasmamembrane of a cell, the enzyme does not couple the hydration or dehydration of carbonic acid to an exergonic reaction and therefore does not change the equilibrium of the C_i species. The theoretical carbon flux based on (uncatalyzed) diffusional CO_2 delivery at the plasmamembrane can be calculated as follows.

In a cell with a radius of 2.5 μm , the diffusional CO_2 influx, Q_a , is given by $Q_a = 4\pi a D_{\text{CO}_2} \Delta[\text{CO}_2]$ (in mol C s^{-1}), where a is the effective boundary layer thickness, which in the spherical cells of microalgae is equal to the radius of that cell, D_{CO_2} is the diffusion coefficient of CO_2 (in seawater at 12°C, $1.481 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$; Wolf-Gladrow and Riebesell 1997). $\Delta[\text{CO}_2]$ is difference between $[\text{CO}_2]_{\infty}$, in the bulk phase and $[\text{CO}_2]_r$, at the plasmamembrane. This formula yields for *Phaeocystis* cells a value for $\Delta[\text{CO}_2]$ of $46.5 \times 10^{-15} \Delta[\text{CO}_2] \text{ mol s}^{-1}$.

In a typical experiment, the C_i influx determined by ^{14}C incorporation was $4.2 \times 10^{-18} \text{ mol s}^{-1} \text{ cell}^{-1}$. Given the amount of carbon per cell of 0.6 pmol cell^{-1} ($0.009 \times \text{cell volume}$; Strathmann 1967), this photosynthetic rate would give a cell doubling rate of 0.6 d^{-1} , which is close to the observed rates. To account for the experimentally observed C flux for *Phaeocystis*, the value for the diffusion gradient $\Delta[\text{CO}_2]$ needs to be only 0.09 μM . So although the concentration difference necessary to drive the diffusive entry of CO_2 at a rate sufficient to support the observed photosynthetic rate is small, the presence of external CA will decrease this concentration difference and thus be beneficial when the external CO_2 concentration gets close to $K_{0.5}$ of Rubisco, the steep region of the dose-response curve.

If no carbon-concentrating mechanism is present in a species, the CO_2 concentration at the pH that is reached in the stationary phase should be close to the compensation concentration for Rubisco of CO_2 . These compensation concen-

trations range in microalgae from 0.195 to 2.88 μM (Falkowski and Raven 1997). In a medium with 3 mM total inorganic carbon, these values correspond to a pH range of 8.8–9.5. This range closely matches the values found for species that show extracellular CA activity. In typical seawater with 2 mM total inorganic carbon, the compensation concentrations would correspond to a pH range of 8.7–9.4. The species that utilize HCO_3^- and are not influenced by AZ reach higher pH values, which could be an indication of the operation of a carbon-concentrating mechanism.

Although the inhibition of CA activity by AZ changes only marginally in the pH range tested (Pocker and Watanori 1973), the inhibition of the steady-state C fixation by AZ shows a distinct pH dependence. The inhibition is 11% at pH 8.4, is maximal at pH 8.6 (22%), and then gradually decreases to 0% at pH 9.2. This dependence can be understood by calculating the theoretical contribution of HCO_3^- conversion to the C supply at the plasmamembrane. This can be done by determining the Damköhler number, which gives a measure of the relative importance of diffusive CO_2 flux and conversion from HCO_3^- for the carbon supply to the cell (see Wolf-Gladrow and Riebesell 1997 and Gavis and Ferguson 1975 for a derivation of the diffusion-reaction equations describing the carbonate system in the vicinity of microalgal cells). The Damköhler number is a dimensionless number representing the ratio of the diffusional time scale and the time scale of the HCO_3^- to CO_2 conversion reaction. As the H^+ concentration is a parameter determining the Damköhler number, its value is pH dependent. For a cell the size of *Phaeocystis*, the uncatalyzed contribution of HCO_3^- conversion is insignificant (it increases from <0.015% at pH 8.2 to 0.45% at pH 9.2). With CA active, in the absence of AZ, conversion of HCO_3^- to CO_2 is 38 times faster in the unstirred layer (see Figs. 2B, 4). This means that at pH 8.2 HCO_3^- conversion still only contributes 0.5% of the CO_2 flux into the cell, but that at pH 9.2 the contribution has increased to about 18%. However, at such a high pH, the concentration of CO_2 has come close to the compensation concentration for Rubisco, giving only a minimal net C fixation. These two processes, the increase of importance of HCO_3^- conversion, and lowering of the net C fixation rate result in the apparent optimum in the inhibition of steady-state ^{14}C fixation by AZ (Fig. 5).

Conclusions

In light of the results of Mercado et al. (1997a) and of the present study, the failure to detect any CA activity in marine microalgae with the potentiometric method does not necessarily mean that extracellular CA activity is not physiologically relevant. A similar conclusion was reached before for marine macroalgae (Haglund et al. 1992)

The induction of extracellular CA activity at low external CO_2 , i.e., *Phaeodactylum* (John-McKay and Colman 1997), several other species (Nimer et al. 1997), and *Phaeocystis*, and the variability in the induction of extracellular CA activity at low CO_2 , i.e., *Phaeodactylum* (John-McKay and Colman 1997) and *E. huxleyi*, implies a high plasticity in the mode of inorganic carbon acquisition of marine algal

species. One should therefore be cautious with the use of fixed parameters for photosynthesis in model studies.

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