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Inorganic carbon acquisition in red tide dinoflagellates

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ABSTRACT

Carbon acquisition was investigated in three marine bloom-forming dinoflagellates – *Prorocentrum minimum*, *Heterocapsa triquetra* and *Ceratium lineatum*. *In vivo* activities of extracellular and intracellular carbonic anhydrase (CA), photosynthetic O_2 evolution, CO_2 and HCO_3^- uptake rates were measured by membrane inlet mass spectrometry (MIMS) in cells acclimated to low pH (8.0) and high pH (8.5 or 9.1). A second approach used short-term ^{14}C-disequilibrium incubations to estimate the carbon source utilized by the cells. All three species showed negligible extracellular CA (eCA) activity in cells acclimated to low pH and only slightly higher activity when acclimated to high pH. Intracellular CA (iCA) activity was present in all three species, but it increased only in *P. minimum* with increasing pH. Half-saturation concentrations ($K_{1/2}$) for photosynthetic O_2 evolution were low compared to ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) kinetics. Moreover, apparent affinities for inorganic carbon (Ci) increased with increasing pH in the acclimation, indicating the operation of an efficient CO_2 concentration mechanism (CCM) in these dinoflagellates. Rates of CO_2 uptake were comparably low and could not support the observed rates of photosynthesis. Consequently, rates of HCO_3^- uptake were high in the investigated species, contributing more than 80% of the photosynthetic carbon fixation. The affinity for HCO_3^- and maximum uptake rates increased under higher pH. The strong preference for HCO_3^- was also confirmed by the ^{14}C-disequilibrium technique. Modes of carbon acquisition were consistent with the ^{13}C-fractionation pattern observed and indicated a strong species-specific difference in leakage. These results suggest that photosynthesis in marine dinoflagellates is not limited by Ci even at high pH, which may occur during red tides in coastal waters.

Key-words: *Ceratium lineatum*; *Heterocapsa triquetra*; *Prorocentrum minimum*; ^{13}C fractionation; CO_2 concentrating mechanism; CO_2 uptake; HCO_3^- uptake; pH; photosynthesis.

INTRODUCTION

Inorganic carbon (Ci) acquisition has been suggested to play an important role in marine phytoplankton ecology and evolution (Tortell 2000; Rost *et al*. 2003; Giordano, Beardall & Raven 2005). Despite the relatively high concentrations of dissolved inorganic carbon (DIC) in marine environments, phytoplankton cells have to invest considerable resources in carbon acquisition to allow for high rates of photosynthesis. This circumstance is mainly caused by the primary carboxylating enzyme, ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), which is restricted to CO_2 for carbon fixation. This highly conserved enzyme is characterized by a low affinity for its substrate CO_2, slow maximum specific turnover rate and susceptibility to a competing reaction with O_2. The latter reaction initiates the process of photorespiration, which further lowers the rate of carbon fixation.

Under present atmospheric conditions (i.e. low CO_2 and high O_2 levels), the catalytic inefficiency of Rubisco imposes restrictions on the carbon assimilation of all photoautotrophs. This is particularly true for algae as CO_2 availability in water is further reduced, owing to slow CO_2 diffusion rate, slow conversion rate between HCO_3^- and CO_2 and low CO_2 concentrations under alkaline conditions. To avoid carbon limitation in photosynthesis, algae developed mechanisms to enhance intracellular CO_2 concentration at the site of carboxylation. These CO_2 concentrating mechanisms (CCMs) involve active uptake of CO_2 and/or HCO_3^-, as well as of the enzyme carbonic anhydrase (CA), which accelerates the otherwise slow conversion rate between HCO_3^- and CO_2. In addition to an effective uptake of Ci, it is equally necessary for microalgae to minimize Ci losses via leakage, a risk that increases with increasing accumulation of Ci. Phytoplankton species differ in efficiency and regulation of their CCMs (e.g. Burkhardt *et al*. 2001; Beardall & Giordano 2002; Rost *et al*. 2003) and in the catalytic efficiency of their Rubisco (Badger *et al*. 1998; Tortell 2000). As a consequence, some species are CO_2-sensitive in their photosynthesis, whereas other species are rate-saturated even under low ambient CO_2 concentrations.

Dinoflagellates are a diverse and abundant group of protists with complex interactions in the food web. They can form so-called 'red tides' in coastal waters, a name that was given because of the changes in water colour observed at times. Such mass development has enormous ecological implications, especially because some dinoflagellates are known to produce various toxins. Despite its ecological and economic importance, relatively little research on photosynthesis and carbon acquisition has been done on this group (Giordano *et al*. 2005). However, this task is very

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intriguing because most dinoflagellates seem to possess the form II Rubisco, featuring one of the lowest affinities for CO_2 and a high sensitivity for O_2 (Badger *et al*. 1998). Only Rubiscos of cyanobacteria shows similarly low substrate-specificity factors (S_{rel}), a parameter describing the selectivity of the carboxylation over oxygenation reaction of Rubisco.

Laboratory experiments demonstrated the capability of dinoflagellates to accumulate Ci relative to ambient concentrations during photosynthesis. Accumulation factors varied between 5- and 70-fold, depending on the species and growth condition (Berman-Frank, Erez & Kaplan 1998; Leggat, Badger & Yellowlees 1999; Nimer, Brownlee & Merrett 1999). Mechanisms that enhance the intracellular CO_2 concentration require active uptake of either or both CO_2 and HCO_3^-. Studies on zooxanthellae isolates from corals found that HCO_3^- is the carbon species mostly taken up (Goiran *et al*. 1996). In isolates from giant clams, the preferred carbon source changed from CO_2 to HCO_3^- after incubation in seawater for 2 d (Leggat *et al*. 1999). A recent study on the free-living dinoflagellates *Amphidinium carterae* and *Heterocapsa oceanica* found no evidence of HCO_3^- use (Dason, Huertas & Colman 2004). Based on their findings, it was suggested that these species are CO_2 limited in their natural environment, even at air-equilibrated CO_2 values.

In coastal waters, photosynthetic activity during dinoflagellate blooms may result in elevated pH levels in the photic zone (Hansen 2002; Hinga 2002). These dinoflagellate blooms last for extended periods of time (several weeks) and the pH level often reaches values above 9 – occasionally up to 9.75 (Hansen 2002). Such high pH levels may cause a succession of phytoplankton species, as some species are more sensitive to elevated pH than others. While species growth in some dinoflagellates is already affected above pH 8.4, other species grow unaffected until pH values reach 10.2 (see Hansen 2002). The reason for this large difference in pH tolerance among dinoflagellates is unknown, but a number of different suggestions have been put forward, including Ci limitation (e.g. Hansen 2002). At high pH, the concentration of DIC is reduced owing to its removal by the algae. As a result of concomitant changes in the chemical speciation, a greater proportion of the Ci pool will be in the form of CO_3^{2-}, which may be unavailable for algae. More importantly, increasing pH causes the CO_2 concentration to drop far below air-equilibrated CO_2 values. Thus, species which rely mostly on CO_2 as a Ci source will be less competitive at high pH.

In the present study, we investigated modes of carbon acquisition of three marine dinoflagellates acclimated to low and high pH. Our test organisms, *Prorocentrum minimum*, *Heterocapsa triquetra* and *Ceratium lineatum*, are all bloom-forming dinoflagellates with different levels of tolerance to high pH. In each species, we examined photosynthetic O_2 evolution and quantified CO_2 and HCO_3^- fluxes across the plasmalemma during steady-state photosynthesis using membrane inlet mass spectrometry (MIMS) (Badger, Palmqvist & Yu 1994). As a second independent technique, short-term ^{14}C-disequilibrium experiments were conducted to estimate the carbon source being utilized by the cells. Activities of extracellular and intracellular CA were determined by monitoring ^{18}O exchange from doubly labelled $^{13}C^{18}O_2$ (Palmqvist, Yu & Badger 1994). In addition, we took samples to analyse the isotopic composition of particulate organic carbon (POC), which allowed us to calculate ^{13}C fractionation (ε_P) of the cells.

MATERIALS AND METHODS

Culture conditions and sampling

P. minimum, *H. triquetra* and *C. lineatum* (isolates from the Marianger Fjord, culture collection of the Marine Biological Laboratory in Helsingør, Denmark) were grown at 15 °C in 0.2 μm filtered and unbuffered seawater (salinity 34), which was enriched with nutrients according to an f/2 medium (Guillard & Ryther 1962). Dilute batch cultures were grown in 2.4 L borosilicate bottles under a light-dark cycle of 16:8 h and an incident photon flux density (PFD) of 150 μmol photons m^{-2} s^{-1}. A light-dark cycle was chosen because continuous light caused much lower rates for photosynthesis and Ci uptake in marine phytoplankton of different taxa (Rost, Riebesell & Sültemeyer 2006).

In the cultures, pH was adjusted by addition of HCl or NaOH to a lower pH of 8.0 and a higher pH of 8.5 or 9.1. This corresponds to CO_2 concentrations of 22.6, 7.1 and 1.4 μmol CO_2 L^{-1}, respectively. The upper pH was chosen based on the pH-dependence of growth of the respective species (i.e. the highest pH at which cell division remains unaltered). This was 9.1 for *P. minimum* and *H. triquetra*; as for *C. lineatum*, growth was already affected above 8.5 (Hansen 2002). Cultures were not bubbled with air that contain different CO_2 partial pressures because dinoflagellates are known to be negatively affected by turbulence. Daily dilution with fresh media ensured that the pH level remained constant (± 0.1 units) and that the cells stayed in the mid-exponential growth phase. Growth rates were about 0.45 d^{-1} in *P. minimum*, 0.55 d^{-1} in *H. triquetra* and 0.35 d^{-1} in *C. lineatum*, both at low and high pH. Cell concentrations in the cultures ranged between 500 and 3000 cells mL^{-1}.

After at least 5 d of acclimation to the respective conditions and within 3–7 h after the beginning of the photoperiod, cells were concentrated by gentle filtration over an 8 μm filter. The culture media was hereby stepwise exchanged with the respective buffered assay media. In case of assays for CA activity or Ci fluxes by MIMS, cells were transferred into a CO_2-free f/2 medium, buffered with 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES, 50 mmol L^{-1}, pH 8.0). Short-term ^{14}C incubations required cells to be transferred into an f/2 medium, buffered with N,N-Bis(2-hydroxyethyl) glycerine (BICINE, 20 mmol L^{-1}, pH 8.5).

Samples for the determination of Chl *a* concentration were taken after the measurements by centrifuging an

aliquot of the cell suspension. Pellets were stored at –80 °C until they were extracted in 1 mL of acetone (overnight in darkness, at –28 °C) and analysed by high-performance liquid chromatography (HPLC). Chl *a* concentrations in the assays ranged from 0.05 to 0.7 μg mL^{-1}.

Determination of CA activity

CA activity was determined from the ^{18}O-depletion of doubly labelled $^{13}C^{18}O_2$ in water caused by several CO_2 and HCO_3^- hydration and dehydration steps (Silvermann 1982). This mass spectrometric procedure allows the determination of CA activity from intact cells under conditions similar to those during growth and differentiates between extracellular CA (eCA) and intracellular CA (iCA) activity (e.g. Rost *et al*. 2003).

All measurements were carried out in an 8 mL thermostated cuvette, which was attached to a sectorfield multicollector mass spectrometer (IsoPrime; GV Instruments, Manchester, UK) via a gas-permeable membrane [polytetrafluoroethylene (PTFE), 0.01 mm] inlet system. Changes in the ion-beam intensities corresponding to concentrations of the CO_2 isotopomers $^{13}C^{18}O^{18}O$ (m/z = 49), $^{13}C^{18}O^{16}O$ (m/z = 47) and $^{13}C^{16}O^{16}O$ (m/z = 45) were recorded continuously and calculated as:

$$^{18}\text{O log(enrichment)} = \log\frac{(^{13}C^{18}O_2)\times 100}{^{13}CO_2} = \log\frac{(49)\times 100}{45+47+49}. \quad (1)$$

Measurements of eCA and iCA activities were performed in the CO_2-free f/2 medium, buffered with HEPES-NaOH (50 mmol L^{-1}, pH 8.0) at 15 °C. All assays were carried out in the dark, unless stated otherwise. $NaH^{13}C^{18}O_3$ was added to a final concentration of 1 mmol L^{-1} and the uncatalysed rate of ^{18}O loss was recorded for at least 8 min. Afterwards, 50–150 μL of cells suspension were added to yield a final Chl *a* concentration of 0.1–0.5 μg mL^{-1}. Representative results for such an assay are given in Fig. 1. For calculation of eCA activity, the linear rate of decrease in ^{18}O-atom fraction after the addition of the sample (S_2) was compared to the non-catalysed decline (S_1) and normalized on a Chl *a* basis (Badger & Price 1989):

$$U = \frac{(S_2 - S_1)\times 100}{S_1 \times \text{mg Chl } a}. \quad (2)$$

iCA activity was estimated from the rapid decline in log(enrichment) upon injection of cells and calculated according to Palmqvist *et al*. (1994). In this assay, a membrane-impermeable inhibitor of CA [dextran-bound sulfonamide (DBS), Synthelec AB, Lund, Sweden] was added prior to the injection of cells to a final concentration of 50 μmol L^{-1}. Subsequent to the iCA measurements, light was switched on (300 μmol photons m^{-2} s^{-1}) to monitor light-induced changes in the ^{18}O exchange.

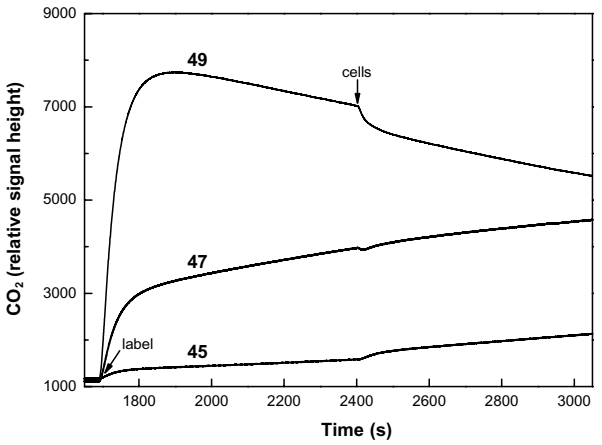

Figure 1. Example for a mass spectrometric carbonic anhydrase (CA) assay showing changes in the relative concentrations of the CO_2 isotopes $^{13}C^{18}O_2$ (m/z = 49), $^{13}C^{16}O^{18}O$ (m/z = 47) and $^{13}CO_2$ (m/z = 45) upon the addition of $NaH^{13}C^{18}O_3$ (1 mmol L^{-1}). The uncatalysed rate of ^{18}O loss was recorded for about 8 min followed by the addition of cells. This graph shows an example for *Prorocentrum minimum* acclimated to pH 9.1.

Isotope-disequilibrium experiments

The ^{14}C-disequilibrium technique makes use of the transient *isotopic* disequilibrium upon an acidic ^{14}C spike into cell suspension at high pH to determine whether CO_2 or HCO_3^- is the preferred carbon species for photosynthesis (Espie & Colman 1986; Elzenga, Prins & Stefels 2000). In the present study we largely followed the protocol described by Tortell & Morel (2002), with a few modifications.

Cells were transferred into a cuvette (4 mL volume) containing an f/2 medium buffered at pH 8.5 (BICINE-NaOH, 20 mmol L^{-1}) at 15 °C. After pre-incubation to 300 μmol photons m^{-2} s^{-1} for 6 min, a 20 μCi ^{14}C spike of pH 7.0 [CFA3 (Amersham Pharmacia Biotech, Cardiff, UK) in HEPES, 50 mmol L^{-1}] was injected into the cell suspension. To examine the importance of eCA, incubations were run without and with DBS (50 μmol L^{-1}). After the injection of the ^{14}C spike, 200 μL subsamples were withdrawn at short intervals and dispensed into 1.5 mL of HCl (6 N). To remove residual inorganic ^{14}C that had not been fixed, samples were purged with air for at least 3 h. Afterwards, 10 mL scintillation cocktail (Ultima Gold AB, PerkinElmer, Boston, MA, USA) was added to the vials and ^{14}C was measured by standard liquid scintillation procedures. To correct for small residual inorganic ^{14}C, blanks consisting of spike added to cell-free buffer were measured.

For quantitative interpretation of ^{14}C-disequilibrium data we fitted the data according to equations derived from Espie & Colman (1986) and Elzenga *et al*. (2000). Briefly, the instantaneous rate of Ci uptake is equal to the sum of CO_2 and HCO_3^- uptake at any time and is given by (Elzenga *et al*. 2000):

$$d(DPM_t)/dt = V_{CO_2} \times SA_{CO_2t} \times V_{HCO_3^-} \times SA_{HCO_3^-t} \quad (3)$$

where $d(DPM_t)/dt$ is the instantaneous rate of Ci uptake at time t and V_{CO_2} and $V_{HCO_3^-}$ are the rates of uptake for CO_2 and HCO_3^-, respectively. The differences in CO_2- and HCO_3^--specific activities affect the instantaneous rate of ^{14}C uptake from these species. SA_{CO_2t} and $SA_{HCO_3^-t}$ are the specific activities of CO_2 and HCO_3^-, respectively. Changes in SA of CO_2 and HCO_3^- with time are given by (Elzenga *et al.* 2000):

$$SA_{CO_2t} = SA_{DIC} + \Delta SA_{CO_2} \times e^{-\alpha_1 t} \text{ and} \quad (4)$$

$$SA_{HCO_3^-t} = SA_{DIC} + \Delta SA_{HCO_3^-} \times e^{-\alpha_2 t}, \quad (5)$$

where SA_{DIC} is the specific activity of the total DIC at equilibrium, ΔSA is the difference between the initial and equilibrium values in the specific activity of CO_2 and HCO_3^-, and α_1 and α_2 are the temperature, salinity and pH-dependent first-order rate constants for CO_2 and HCO_3^-, respectively, as described by Espie & Colman (1986). Our experimental constants were: temperature = 15 °C; salinity = 34; pH value = 8.5; $\alpha_1 = 0.0272$; and $\alpha_2 = 0.0315$. α_1 and α_2 were determined by adjusting temperature and salinity corrections acquired from Johnson (1982). The integrated accumulation of ^{14}C has been modified from Elzenga *et al.* (2000) by introducing f as the proportion of HCO_3^- relative to net Ci fixation:

$$DPM_t = V(1-f) \times [\alpha_1 t + (\Delta SA_{CO_2} / SA_{DIC}) \times (1 - e^{-\alpha_1 t})]/\alpha_1 + V(f) \times [\alpha_2 t + (\Delta SA_{HCO_3^-} / SA_{DIC}) \times (1 - e^{-\alpha_2 t})]/\alpha_2. \quad (6)$$

The values of $\Delta SA_{CO2}/SA_{DIC}$ and $\Delta SA_{HCO3}/SA_{DIC}$ are set by the difference in pH between the ^{14}C spike and seawater buffer, with values of 49 and –0.24, respectively.

Determination of net photosynthesis and Ci fluxes

The mass spectrometric technique established by Badger *et al.* (1994) uses the *chemical* disequilibrium between CO_2 and HCO_3^- during light-dependent Ci uptake to differentiate between CO_2 and HCO_3^- fluxes across the plasmalemma. It is based on simultaneous measurements of O_2 and CO_2 during consecutive light and dark intervals. During dark intervals, known amounts of Ci were added to measure rates as a function of CO_2 and HCO_3^- concentrations. As for all disequilibrium techniques, a lack of eCA activities is required.

Estimates of the O_2, CO_2 and HCO_3^- fluxes were made using equations of Badger *et al.* (1994). Briefly, rates of O_2 consumption in the dark and O_2 production in the light were used as direct estimates of respiration and net Ci fixation, assuming a respiratory quotient of 1.0 and a photosynthetic quotient of 1.1 to convert O_2 fluxes into Ci fluxes. Net CO_2 uptake was calculated from the steady-state rate of CO_2 depletion at the end of the light period and corrected for the CO_2/HCO_3^- interconversion in the medium. The HCO_3^- uptake was derived by a mass balance equation (i.e. the difference of net Ci fixation and net CO_2 uptake). The pseudo-first-order rate constant k_2 (formation of CO_2 from HCO_3^-) was determined experimentally from the initial slope of CO_2 evolution after injection of known amounts of HCO_3^- into a CO_2-free buffered medium. The rate constant k_1 (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. CO_2 efflux was estimated from the initial CO_2 increase observed directly after the transition from light to dark.

In the present experiments, light/dark intervals during the assay lasted 6 and 7 min, respectively. All measurements were performed in an f/2 medium, buffered with HEPES (50 mmol L^{-1}, pH 8.0) at 15 °C. The incident PFD was 300 μmol m^{-2} s^{-1}. DBS concentration was 50 μmol L^{-1} in order to ensure the complete inhibition of any eCA activity. Rate constants k_1 and k_2 were determined daily in the freshly prepared assay medium, yielding mean values of 1.08 (± 0.08) min^{-1} and 2.1 (± 0.2) × 10^{-2} min^{-1}, respectively. Chl *a* concentrations in the assays ranged between 0.1 and 0.7 μg mL^{-1}.

Isotope fractionation

Samples for POC were filtered onto pre-combusted QMA filters (Whatman International Ltd, Maidstone, UK) (500 °C; 12 h) and stored at –25 °C in pre-combusted Petri dishes (500 °C; 12 h). Prior to the measurement, POC filters were treated with 200 μL HCl (0.1 N) to remove all Ci and afterwards dried for 2 h at 60 °C. POC and related $\delta^{13}C$ values were measured in duplicate on an EA mass spectrometer (ANCA-SL 20–20, Sercon Ltd, Crewe, UK), with a precision of ± 1.5 μg C and ± 0.5‰, respectively. The isotopic composition is reported relative to the PeeDee belemnite standard (PDB):

$$\delta^{13}C_{sample} = \left[\frac{(^{13}C/^{12}C)_{Sample}}{(^{13}C/^{12}C)_{PDB}} - 1 \right] \times 1000. \quad (7)$$

Isotope fractionation during POC formation (ε_P) was calculated relative to the isotopic composition of CO_2 in the medium (Freeman & Hayes 1992):

$$\varepsilon_p = \frac{\delta^{13}C_{CO_2} - \delta^{13}C_{POC}}{1 + \frac{\delta^{13}C_{POC}}{1000}}. \quad (8)$$

To determine isotopic composition of DIC ($\delta^{13}C_{DIC}$), 8 mL of the culture medium was fixed with HgCl (final concentration, 140 mg L^{-1}). Extractions and measurements were performed in the laboratory of H. J. Spero, University of California, Davis, with a precision of ± 0.11‰. The isotopic composition of CO_2 ($\delta^{13}C_{CO_2}$) was calculated from $\delta^{13}C_{DIC}$, making use of a mass balance relation (see Zeebe & Wolf-Gladrow 2001):

$$\delta^{13}C_{HCO_3^-} = \frac{\{\delta^{13}C_{DIC}[DIC] - (\varepsilon_a[CO_2] + \varepsilon_b[CO_3^{2-}])\}}{\{(1 + \varepsilon_a \times 10^{-3})[CO_2] + [HCO_3^-] + (1 + \varepsilon_b \times 10^{-3})[CO_3^{2-}]\}} \quad (9)$$

$$\delta^{13}C_{CO_2} = \delta^{13}C_{HCO_3^-}(1 + \varepsilon_a \times 10^{-3}) + \varepsilon_a. \quad (10)$$

Temperature-dependent fractionation factors between CO_2 and HCO_3^- (ε_a) as well as HCO_3^- and CO_3^{2-} (ε_b) are given by Mook (1986) and Zhang, Quay & Wilbur (1995), respectively.

RESULTS

CA activity

Determination of CA activity using MIMS distinguishes between eCA and iCA activity. Figure 1 shows a representative example for such an assay. The initial increase in the different CO_2 traces is due to the label addition. The ^{18}O-loss of doubly labelled HCO_3^- prior and after the addition of cells did not significantly differ, indicating no or only little eCA activity in *P. minimum*. Growth under higher pH induced slightly higher activities in *P. minimum* (Table 1). In *H. triquetra* and *C. lineatum*, eCA activities were close to the detection limit and remained unaffected by the pH in the incubation. iCA activities were low in all three species and only in *P. minimum* did it respond to changes in pH. The ^{18}O-exchange technique also indicates the presence of light-dependent Ci transport systems. As shown in Fig. 2, illumination of *H. triquetra* resulted in a faster uptake of ^{18}O-labelled $^{13}CO_2$ (m/z = 49 and 47) and consequent efflux of unlabelled $^{13}CO_2$ (m/z = 45), leading to a light-dependent decrease in log(enrichment). While similar results were obtained for *P. minimum*, illumination induced a different pattern of ^{18}O exchange in *C. lineatum*. In the latter, light-stimulation in the ^{18}O exchange was less pronounced and observed only in cells acclimated to high pH. Moreover, there was a transient increase in the log(enrichment) shortly after light was turned on.

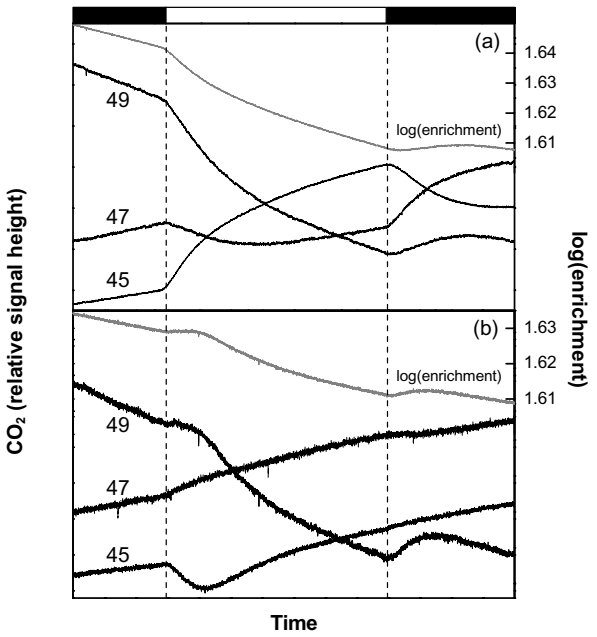

Figure 2. Time course of changes in the relative concentrations of the CO_2 isotopes $^{13}C^{18}O_2$ (m/z = 49), $^{13}C^{16}O^{18}O$ (m/z = 47), $^{13}CO_2$ (m/z = 45) and the ^{18}O log (enrichment) by cells of *Heterocapsa triquetra* (a) and *Ceratium lineatum* (b). Subsequent to the carbonic anhydrase (CA) measurements, the extracellular CA (eCA) inhibitor dextran-bound sulfonamide (DBS; 50 μmol L^{-1}) was applied and light was turned on for 4 min (300 μmol photons m^{-2} s^{-1}). Black and white bars at the top indicate the dark and light period, respectively. The figure shows representative data from cells acclimated to high pH. The pattern of the ^{18}O exchange in *Prorocentrum minimum* (not shown) was similar to those for *H. triquetra*.

Table 1. Chl *a*-specific activities of extracellular carbonic anhydrase (eCA) and intracellular carbonic anhydrase (iCA) activities from cells acclimated to low and high pH. Values represent the mean of three independent measurements (± SD)

	CA activity	
pH	eCA [U (mg Chl *a*)$^{-1}$]	iCA [Δ (mg Chl *a*)$^{-1}$]
Prorocentrum minimum		
8.0	47 ± 15	16 ± 2
9.1	199 ± 24	24 ± 1
Heterocapsa triquetra		
8.0	13 ± 3	8 ± 2
9.1	26 ± 10	4 ± 1
Ceratium lineatum		
8.0	25 ± 14	32 ± 8
8.5	34 ± 2	29 ± 1

CA, carbonic anhydrase.

^{14}C-disequilibrium technique

The rate of ^{14}C incorporation was monitored over at least 12 min, with emphasis on the first 30 s and the last 8 min. Monitoring the ^{14}C incorporation well into the equilibrium yielded a high level of precision for determining the contribution of HCO_3^-. Figure 3 shows an example for the ^{14}C incorporation of *P. minimum*, acclimated to pH 8.0. Fitting the data yielded a high contribution of HCO_3^- relative to net fixation, about 80% in the given example. When repeated without DBS (control), similar rates of ^{14}C incorporation were obtained, indicating direct uptake of HCO_3^- and the absence of significant eCA activity. The latter has already been shown from the ^{18}O exchange. It also indicated that neither the process of concentrating by filtration nor DBS affected the cells negatively. In all three species, the overall proportion of HCO_3^- makes up about 85% of the total carbon fixed (Table 2, Fig. 4). In *P. minimum*, the preference for HCO_3^- even increases under higher pH, where it contributes about 94%.

Photosynthesis and Ci fluxes

The mass spectrometric approach by Badger *et al.* (1994) is based on simultaneous O_2 and CO_2 measurements during consecutive light/dark intervals. Figure 5 shows a typical time course of O_2 and CO_2 concentrations with increasing photosynthetic O_2 production and CO_2 consumption in the light. After about 3 min, the rate of O_2 evolution and CO_2

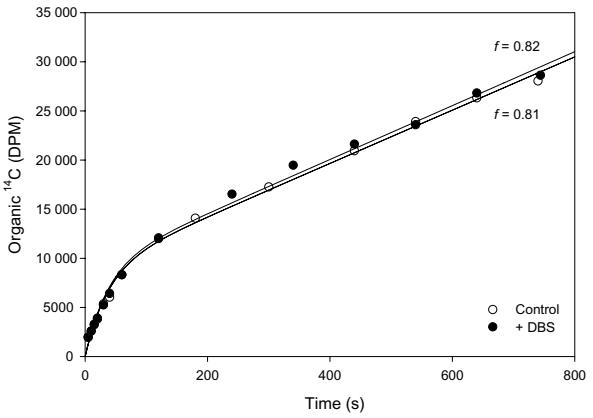

Figure 3. Examples of the results of short-term ^{14}C incubations with cells of *Prorocentrum minimum*, acclimated to pH 8.0. Values of *f* represent the proportion of HCO_3^- uptake relative to net C-fixation in dextran-bound sulfonamide (DBS)-treated cells (50 μmol L^{-1}) and the control. Comparison of DBS-treated cells with the control allows differentiating between direct HCO_3^- *uptake* and CA-mediated HCO_3^- *utilization*.

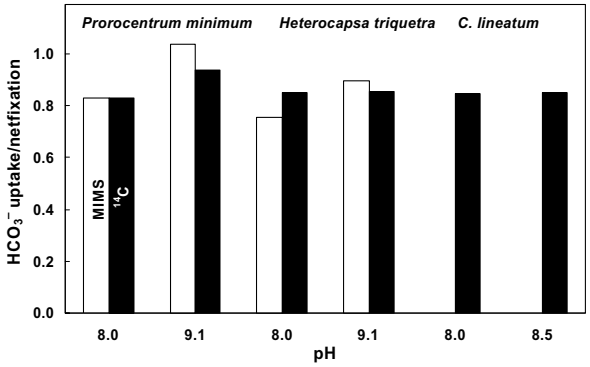

Figure 4. Contribution of HCO_3^- uptake relative to net photosynthesis determined by inorganic carbon (Ci)-flux measurements (white columns) and ^{14}C disequilibrium technique (black columns) in cells acclimated to different pH. Ratios from membrane inlet mass spectrometry (MIMS) measurements were based on the rates obtained at Ci concentrations of about 2 mmol L^{-1}. Ci-flux measurements were not obtained for *C. lineatum* because the cells were affected by stirring over the duration of the assay.

depletion reached a steady state. When the light was turned off, photosynthetic O_2 production ceased immediately and was replaced by respiratory O_2 consumption. The rapid CO_2 increase was caused by both re-equilibration and CO_2 efflux. Based on such changes in O_2 and CO_2 concentration rates of net photosynthesis, CO_2 and HCO_3^- uptake were calculated and expressed as a function of CO_2 and/or HCO_3^- concentration. Representative results of an assay are shown for low- and high-pH-acclimated cells of *H. triquetra* (Fig. 6). The corresponding kinetic parameters of apparent half-saturating concentrations ($K_{1/2}$) and maximum rates (V_{max}) are summarized in Fig. 7. *C. lineatum* was affected by the impeller in the cuvette over the duration of the assay and hence was not included in the following comparison.

In terms of the V_{max} of photosynthesis, *P. minimum* and *H. triquetra* were quite similar and the acclimation pH had

Table 2. Contribution of HCO_3^- uptake relative to net carbon fixation. Values represent the mean of three generally independent measurements (± SD)

	Fraction HCO_3^-	
pH	DBS	Control
Prorocentrum minimum		
8.0	0.83 ± 0.022	0.81 ± 0.024
9.1	0.94	0.92
Heterocapsa triquetra		
8.0	0.85 ± 0.002	0.92 ± 0.016
9.1	0.86 ± 0.002	0.92 ± 0.012
Ceratium lineatum		
8.0	0.85 ± 0.017	0.84 ± 0.028
8.5	0.85 ± 0.005	0.85 ± 0.021

DBS, dextran-bound sulfonamide.

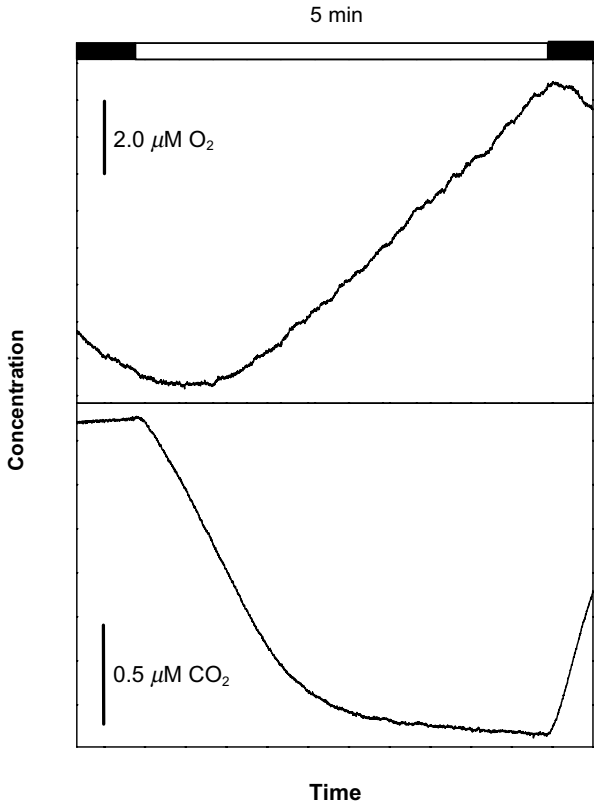

Figure 5. Examples for simultaneous measurements of O_2 and CO_2 concentrations used to estimate rates of photosynthesis, CO_2 uptake and HCO_3^- uptake. Data shown in this figure were obtained with *Heterocapsa triquetra* acclimated to pH 8.0. Black and white bars at the top indicate the dark and light periods, respectively.

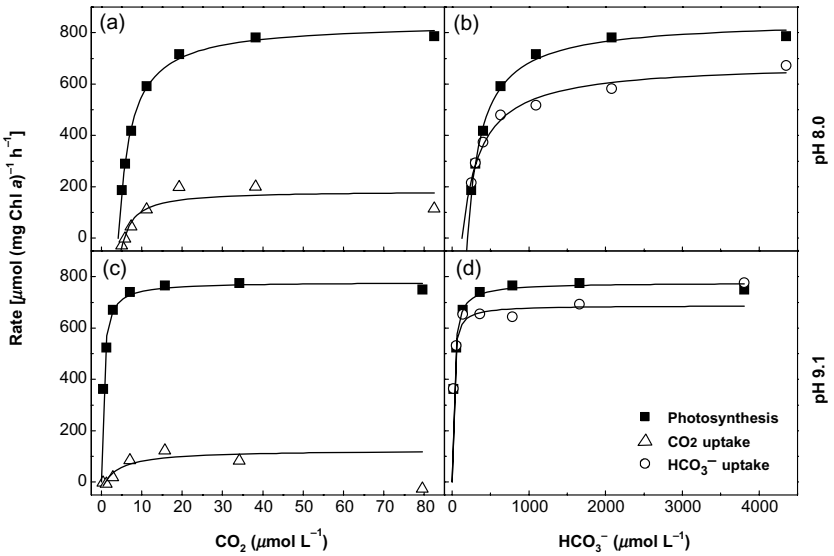

Figure 6. Chl *a*-specific rates of net photosynthesis (squares), net CO_2 uptake (triangles) and HCO_3^- uptake (circles) as a function of CO_2 or HCO_3^- concentration in the assay medium for *Heterocapsa triquetra*. Prior to the measurements, cultures were acclimated to pH 8.0 (a, b) and pH 9.1 (c, d). Curves were obtained from a Michaelis–Menten fit.

no large effect (Fig. 7). As indicated by their $K_{1/2}$ values, affinities for Ci differed significantly between species and acclimations. *P. minimum* showed lower apparent $K_{1/2}$ (HCO_3^-) for photosynthesis than *H. triquetra* when acclimated to pH 8.0. Acclimation to high pH caused these values to decrease strongly, and thus, the differences between the two species diminished. For both species, $K_{1/2}$ (CO_2) for photosynthesis were much lower than could be expected from diffusive CO_2 uptake alone. With values between 0.5 and 8.1 μmol L^{-1} CO_2, this is at least one order of magnitude lower than K_M values reported for Rubisco (Badger *et al*. 1998).

Rates of CO_2 uptake were very low in both species, especially when compared with rates of carbon fixation (Fig. 7). In fact, net CO_2 uptake was even negative for *P. minimum* at times, which made it impossible to calculate $K_{1/2}$ values. In *H. triquetra*, $K_{1/2}$ values for CO_2 uptake were 11.3 and 3.1 μmol L^{-1} CO_2 when grown at pH 8.0 and 9.1, respectively. Because CO_2 uptake could not support the observed rates of photosynthesis, most of the Ci was taken up as HCO_3^-. The contribution of HCO_3^- uptake relative to total carbon fixation was more than 80%, even under low pH (Figs 4 & 7). $K_{1/2}$ values for HCO_3^- uptake were generally lower for *P. minimum* than for *H. triquetra*. For both species, $K_{1/2}$ values strongly decreased with increasing pH; from 79 to 7 μmol L^{-1} HCO_3^- for *P. minimum* and from 426 to 26 μmol L^{-1} HCO_3^- for *H. triquetra*.

The leakage of the cells (i.e. the proportion of Ci efflux compared to gross Ci uptake), was estimated from the CO_2 efflux observed directly upon darkening (Badger *et al*. 1994). As shown in Fig. 8, cellular leakage differed between species, being highest in *C. lineatum* and lowest in *H. triquetra*. In cells acclimated to low pH, leakage was always highest under low ambient CO_2 and levelled off towards higher CO_2 concentrations in the assay. Moreover, leakage seemed to decrease with increasing pH in the incubation.

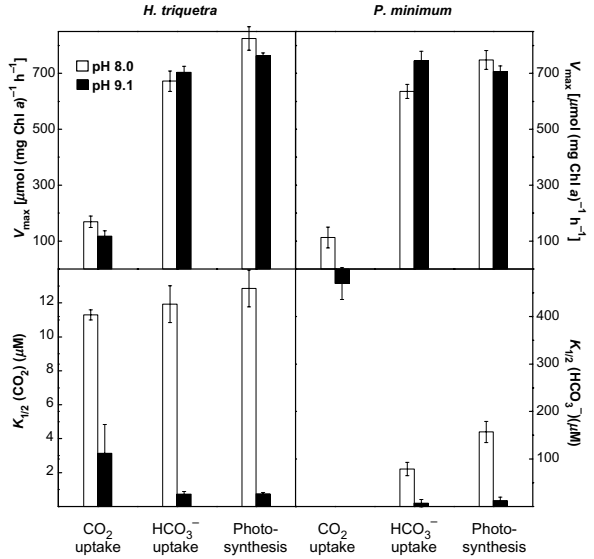

Figure 7. Maximum rates (V_{max}) and half-saturation concentrations ($K_{1/2}$) of photosynthesis, net CO_2 uptake and HCO_3^- uptake for *Heterocapsa triquetra* and *Prorocentrum minimum* acclimated to pH 8.0 (white columns) and pH 9.1 (black columns). With the exception of the CO_2 uptake in *H. triquetra*, $K_{1/2}$ values are reported as μmol HCO_3^- L^{-1}. Kinetic parameters were calculated from a Michaelis–Menten fit to the combined data of several independent measurements. Error bars denote ± SD ($n = 3$).

Isotope fractionation

Samples from each species and pH incubation were taken to determine the isotopic composition of organic carbon ($\delta^{13}C_{POC}$) and Ci ($\delta^{13}C_{DIC}$), allowing us to calculate ^{13}C fractionation of the cells. Fractionation values (ε_P) obtained in this study were generally low, with highest values of 14.4‰ in *C. lineatum*, 9.9‰ in *H. triquetra* and 8.6‰ in *P. minimum* (Fig. 9). In all species, a higher pH in the

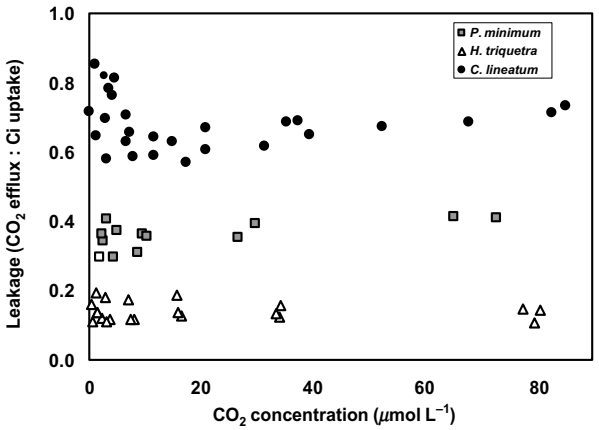

Figure 8. Leakage [CO_2 efflux : gross inorganic carbon (Ci) uptake] as a function of the CO_2 concentration in the assay, obtained from cells of *Prorocentrum minimum*, *Heterocapsa triquetra* and *Ceratium lineatum* acclimated to high pH.

acclimation caused ε_P to decrease by 5 to 10‰, even yielding negative ε_P values (–1.1‰) in *P. minimum*. Such low ε_P values were consistent with predominant HCO_3^- use. Observed ε_P differences between low and high pH, moreover, reflected changes in leakage.

DISCUSSION

Recent studies suggest that photosynthesis and growth in marine dinoflagellates may be CO_2 limited in the natural environment even at air-equilibrated levels found in open waters (Colman *et al*. 2002; Dason *et al*. 2004). In productive coastal waters, Ci limitation may be more severe during algal blooms when the pH is high. In this study, several aspects of Ci acquisition in three bloom-forming dinoflagellates were investigated by applying different independent methods, such as MIMS techniques for CA activity and Ci fluxes, as well as the ^{14}C-disequilibrium technique. The central aim was to describe modes of carbon acquisition under conditions that match the natural environment as much as possible.

CA activity

eCA, which accelerates the conversion of HCO_3^- to CO_2 at the cell surface, was found to increase in response to decreasing CO_2 concentrations or increasing pH in many microalgae (Sültemeyer 1998 and references therein). It is a common notion that eCA increases the CO_2 concentration at the plasma membrane and herewith favours CO_2 uptake, also referred to as *indirect* HCO_3^- utilization. Other microalgal species were found not to induce eCA even when grown under severe carbon limitation (e.g. Burkhardt *et al*. 2001; Rost *et al*. 2003). In the present study, eCA activities were very low in all treatments (Table 1) and only in *P. minimum* did DBS addition cause these activities to considerably decrease (data not shown). The absence of significant eCA activities has also been demonstrated by the ^{14}C-disequilibrium technique, which yielded similar rates of ^{14}C incorporation in DBS-treated, as well as in non-treated, cells. Although some eCA activity could be detected by monitoring ^{18}O loss from labelled HCO_3^-, these activities are negligible when compared to other species. *P. minimum* showed the highest values and only in this species did pH have a noteworthy effect on eCA activity. When grown at pH 8.0 and 9.1, eCA activities were 50 and 200 U (mg Chl *a*)$^{-1}$, respectively. In other words, the rate of interconversion between HCO_3^- and CO_2 increased 0.5- to 2-fold relative to the uncatalysed rate per milligram Chl *a*. Activities observed in other taxa, such as the diatom *Skeletonema costatum* or the prymnesiophyte *Phaeocystis globosa*, were at least one order of magnitude higher under similar growth conditions (Rost *et al*. 2003). Based on these findings, it seems that eCA is playing only a minor role, if any, in the three investigated dinoflagellates.

The low eCA activities observed in the present study are consistent with results of other studies on dinoflagellates. Instead of monitoring ^{18}O loss by mass spectrometry, most other studies on dinoflagellates used a potentiometric approach to assess CA activities (Wilbur & Anderson 1948). Using this approach Dason *et al*. (2004) measured eCA activities in *Amphidinium carterae* and *Heterocapsa oceanica*, yielding Wilbur-Anderson units of 5.05 ± 3.29 and 4.34 ± 1.29 (mg Chl *a*)$^{-1}$, respectively. These eCA activities correspond to a four- to fivefold enhancement in the conversion of HCO_3^- to CO_2 relative to the uncatalysed rate per milligram Chl *a*. These values are within the same magnitude as our results. Nimer, Iglesias-Rodriguez & Merrett (1997) investigated various species, including five species of dinoflagellates, finding relatively low eCA activities in all species and acclimations. In the fresh water dinoflagellates *Peridinium gatunense*, eCA activities were low at the onset of the bloom, but increased up to 570 U (mg Chl *a*)$^{-1}$ towards the end of the bloom (Berman-Frank *et al*. 1995). It should be noted here that the mass spectrometric procedure allows the determination of CA activity from living cells under conditions similar to those during growth. As pointed out by Dason *et al*. (2004), the potentiometric assay may yield erroneous eCA activities owing to low assay

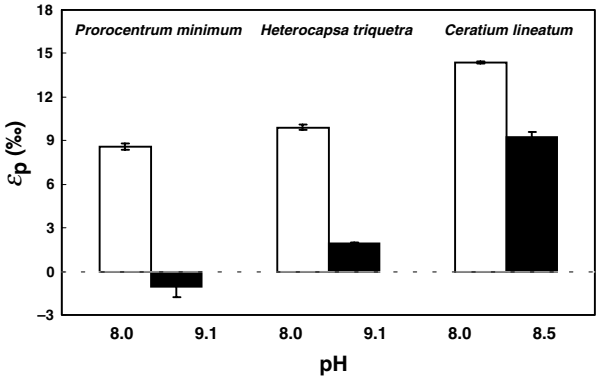

Figure 9. Isotope fractionation (ε_p) as a function of pH in the acclimation, calculated from the $^{13}C_{CO2}$ and $^{13}C_{POC}$ in their respective acclimation. Error bars denote ± SD ($n = 3$).

temperatures and the use of a dilute buffer, which can cause an osmotic shock and, hence, the release of some internal CA.

The physiological role of iCA, which can be located in the chloroplast, the mitochondria and in the cytosol, is still not fully understood (Sültemeyer 1998). The importance of iCA in Ci acquisition has, however, been demonstrated in various studies by the effect of the membrane-permeable CA inhibitor, ethoxyzolamide, on photosynthesis (Badger *et al*. 1998 and references therein). According to Palmqvist *et al*. (1994), when interpreting iCA activities, one has to bear in mind that Δ values are *in vivo* estimates and are dependent not only on the speed of intracellular ^{18}O-depletion but also on the diffusive influx of doubly labelled CO_2, and thus, on the diffusive properties of algal membranes and cell shape. Consequently, Δ values are arbitrary units which allow direct comparison of different treatments, but not necessarily between species. All three species in this investigation possess internal CA activity. Results of Burkhardt *et al*. (2001) indicate a gradual increase in iCA activity of two marine diatoms in response to increasing pH or decreasing CO_2 supply. Our data does confirm such pH dependence in iCA activity only for *P. minimum*.

Using the ^{18}O-exchange technique, we also examined the presence of light-dependent Ci transport systems. In the case of active Ci uptake, a decline in log(enrichment) during illumination would be expected, which is caused by an enhanced influx of ^{18}O-labelled CO_2 and HCO_3^- into the cells to the active site of iCA, increased ^{18}O loss and subsequent efflux of ^{18}O-unlabelled CO_2 (Badger & Price 1989; Palmqvist *et al*. 1994). Such a net CO_2 efflux from photosynthesizing cells result from the accumulation of CO_2 inside the cell relative to ambient CO_2 concentration. As shown in Fig. 2, illumination of *H. triquetra* resulted in an uptake of $^{13}C^{18}O_2$ ($m/z = 49$) and an efflux of $^{13}C^{16}O_2$ ($m/z = 45$), that is, a light-dependent decrease in log(enrichment). Similar ^{18}O exchange was also observed for *P. minimum* (data not shown). These responses to illumination have also been observed in the symbiotic dinoflagellates *Symbiodinium* (Badger *et al*. 1998; Leggat *et al*. 1999), various other microalgae (Palmqvist *et al*. 1994, 1995; Badger *et al*. 1998) and cyanobacteria (Badger & Price 1989), all of which operate a CCM. In *C. lineatum*, light-stimulation in ^{18}O exchange was less pronounced, with a transient increase in the log(enrichment) shortly after light was turned on. The greater lag phase could be explained by a slower induction of the Ci uptake in this species, which is leading to a situation right after illumination in which external Ci species are in equilibrium with the internal compartment. When light activates photosynthesis under these conditions, there is a competition between Rubisco and Ci hydration processes for CO_2, causing the enrichment to rise rather than to fall (see Badger *et al*. 1998).

Photosynthetic O_2 evolution

In comparison to other taxa, dinoflagellates are known to be the most sensitive to turbulence (Thomas & Gibson 1990). We therefore tried to minimize the shear forces on the cells during the measurements by optimizing the stirring mechanism in the cuvette and working with relatively low stirring speed. Time-course experiments confirmed that rates for photosynthesis of *P. minimum* and *H. triquetra* remained unaltered over the duration of the assay. In fact, V_{max} of photosynthesis in these species (Fig. 7) were relatively high when compared to previous studies on dinoflagellates (e.g. Goiran *et al*. 1996; Leggat *et al*. 1999; Nimer *et al*. 1999; Dason *et al*. 2004). Time-course experiments with *C. lineatum*, however, showed that rates of photosynthesis decreased by as much as 40% over the duration of the assay. Consequently, *C. lineatum* is not included in the following comparison on the kinetics of photosynthesis and Ci uptake.

Monitoring photosynthetic O_2 evolution as a function of CO_2 concentration in the assays provides important information on the carbon acquisition of microalgae. Lower apparent $K_{1/2}$ (CO_2) than K_M (CO_2) of Rubisco suggests the operation of a CCM (Badger *et al*. 1998). Treatment-induced changes in affinities bear further more information on the efficiency and capability to regulate the CCM. In our experiments, $K_{1/2}$ (CO_2) for photosynthesis were very low when compared to values known for Rubisco, especially in comparison to type II Rubisco (Whitney & Andrews 1998). *P. minimum* and *H. triquetra* both responded to high pH by increasing the apparent affinities for Ci. In *P. minimum*, $K_{1/2}$ (CO_2) of photosynthesis decreased from 3.2 to 1.6 μmol L^{-1} with increasing pH in the acclimation. In *H. triquetra*, acclimation to high pH had a stronger effect on the up-regulation of the CCM, causing $K_{1/2}$ (CO_2) to drop from 8.1 to 0.5 μmol L^{-1} when grown at pH 8.0 and 9.1, respectively. These affinities are comparable with previous observations, for instance $K_{1/2}$ (CO_2) of *Symbiodinium* sp. being smaller than 3 μmol L^{-1} (Caperon & Smith 1978). In *P. gatunense*, similar changes in affinities as a function of pH were observed, as $K_{1/2}$ (CO_2) for photosynthesis decreased from 4 to 0.1 μmol L^{-1} when grown at pH 8.3 and 9.1, respectively (Berman-Frank *et al*. 1998).

Such low $K_{1/2}(CO_2)$ of photosynthesis relative to the $K_M(CO_2)$ of Rubisco suggest high accumulation of CO_2 at the site of Rubisco. The K_M:$K_{1/2}$ ratio approximates for such an internal CO_2 enrichment relative to ambient CO_2 concentrations. Due to the unstable nature of dinoflagellates Rubisco absolute kinetic parameters are not yet known. However, some estimates can be made based on the S_{rel} values obtained for *A. carterae* (Whitney & Andrews 1998). Assuming a K_M of 60 μmol L^{-1} (Leggat *et al*. 1999), the K_M:$K_{1/2}$ ratio indicate a CO_2 accumulation of about 19- to 37-fold in *P. minimum*. In *H. triquetra*, $K_{1/2}$ (CO_2) values could theoretically be achieved by a CO_2 accumulation of about 7- to 120-fold.

Intracellular accumulation of Ci has been directly measured in dinoflagellates by silicon-oil centrifugation. Leggat *et al*. (1999) observed similar Ci accumulation of about 5- to 25-fold in *Symbiodinium* sp. and *A. caterae*, while Berman-Frank *et al*. (1998) calculated 5- to 70-fold Ci accumulation for *P. gatunense*. In *Prorocentrum micans*,

a 10-fold Ci accumulation relative to external concentration was measured (Nimer *et al*. 1999). These Ci accumulation factors in dinoflagellates obtained by silicon-oil centrifugation seem low in comparison to K_M:$K_{1/2}$ ratios, which indicate rather high CO_2 accumulation at Rubisco. This finding may point to a localized Ci accumulation in the chloroplast, such as the stroma rather than of the whole cell. More accurate estimates of internal CO_2 accumulation will require full kinetic characterization of dinoflagellate Rubisco.

Carbon source and uptake kinetics

Although CO_2 dependence in O_2 evolution reveals information about the efficiency and regulation of the CCM, it can not provide any details about the underlying mechanisms such as the transport systems. Various methods have been employed to distinguish between CO_2 and HCO_3^- uptake in microalgae. In this study, estimates of net CO_2 and HCO_3^- uptake rates were obtained by a method of Badger *et al*. (1994), which has the advantage of having the capability to quantify Ci fluxes during steady-state photosynthesis. Rates of CO_2 uptake were very low in both species, representing less than 20% relative to carbon fixation in all acclimations (Fig. 7). Owing to the low contribution of CO_2 to the overall carbon fixation, $K_{1/2}$ values could not be calculated for *P. minimum*. In *H. triquetra* apparent affinities for CO_2 increased with pH. Both species caused the CO_2 concentration to decrease below disequilibrium concentrations in the light, which has been confirmed by the addition of bovine CA (data not shown). Because CO_2 uptake could not support the observed rates of photosynthesis, most of the Ci was taken up as HCO_3^-. In both species, HCO_3^- uptake contributed more than 80% of the total carbon fixation. Apparent affinities for HCO_3^- were generally higher for *P. minimum* than for *H. triquetra*. Acclimation to high pH caused a strong increase in apparent affinities for HCO_3^-, indicated by the generally lower $K_{1/2}$ values.

In a second approach, we applied the ^{14}C-disequilibrium technique to gain independent information on the carbon source taken by the cells (Espie & Colman 1986; Elzenga *et al*. 2000). This technique has been applied in field studies because the assay can be performed with the lowest cell concentrations, requires no calibration and takes a relatively short time (Tortell & Morel 2002; Cassar *et al*. 2004). Moreover, stirring in the cuvette can be slow, and hence, allow measurements of very sensitive species like *C. lineatum*. It should be noted, however, that neither rates nor affinities for CO_2 or HCO_3^- uptake can be calculated by this approach. The proportion of HCO_3^- relative to carbon fixation was high in all three species, making up between about 83 and 95% (Table 2). Comparing the relative contribution of HCO_3^- obtained by this approach with those obtained by Ci-flux measurements (MIMS), we get similar results (Fig. 4). This assures HCO_3^- as the dominant carbon source taken up.

Our findings contradict with a recent study on free-living dinoflagellates, which found no evidence for HCO_3^- use in *A. carterae* and *H. oceanica* (Dason *et al*. 2004). Their conclusion is mainly based on rather low rates of photosynthetic O_2 evolution in comparison to the spontaneous rate of CO_2 formation. We argue that while rates of photosynthesis higher than the spontaneous CO_2 delivery from the HCO_3^- pool may indicate HCO_3^- use, the reverse conclusion is not valid. In addition, rates of photosynthesis in Dason *et al*. (2004) could be suppressed by photoinhibition, as the cells were acclimated to 75 μmol photons m^{-2} s^{-1}, while rates of photosynthesis were assessed at 1000 μmol photons m^{-2} s^{-1}. A preference for CO_2 in dinoflagellates was also deduced from the stimulation of photosynthesis upon CA addition (Dason *et al*. 2004) or the inhibition of photosynthesis upon DBS addition (Nimer *et al*. 1999). None of these effects were observed in our study. CA addition abolished chemical disequilibrium in the light and hereby increased CO_2 concentrations relative to Ci, but it had no effect on the net photosynthesis (data not shown). Addition of DBS did not alter rates of photosynthesis either (see, for instance, ^{14}C-incorporation rates with and without DBS in Fig. 3).

Fractionation and leakage

Photosynthetic carbon fixation discriminates against the heavier $^{13}CO_2$ causing the isotopic composition of organic material ($\delta^{13}C_{POC}$) to be depleted in ^{13}C, compared with the Ci source. Most of this fractionation (ε_p) is driven by the discrimination of ^{13}C by Rubisco (ε_f), here assumed to be about 30‰ (Raven & Johnston 1991). While ε_f sets the uppermost values for ε_P, variations are principally determined by Ci leakage (L) and the carbon source taken up (Sharkey & Berry 1985):

$$\varepsilon_P = a \times \varepsilon_s + L \times \varepsilon_f. \quad (11)$$

ε_S represents the equilibrium discrimination between the carbon sources CO_2 and HCO_3^- (approx –10‰) and a is the fractional contribution of HCO_3^- to total Ci uptake. Because HCO_3^- is enriched in ^{13}C relative to CO_2, an increasing proportion of HCO_3^- uptake decreases the apparent isotope fractionation ε_p, which is defined relative to CO_2 as the carbon source. If there is no change in the Ci source, ε_p decreases with decreasing leakage. Based on these simple considerations, fractionation values may provide information on the mode of carbon acquisition and vice versa.

In terms of information on the carbon source, only extreme ε_p values allow precluding one carbon source. If ε_p is lower than 0‰, CO_2 can be excluded as the only carbon source and ε_p values higher than 20‰ rule out HCO_3^- as the only carbon source. ε_P values in our experiments (Fig. 9) are within this range, which is consistent with predominant HCO_3^- use. The negative ε_P values of –1.1‰ in *P. minimum* even precluded CO_2 as the only carbon source. Berman-Frank *et al*. (1998) observed high $\delta^{13}C_{POC}$ values (–23 to –16‰) in *P. gatunense*, indicating low fractionation values in this species. Variations in fractionation are particular sensitive to changes in leakage (see Eqn 11). In all species

investigated, high pH caused ε_P to drop by 5–10‰. As HCO_3^- is the predominant carbon source under both pH acclimations (Fig. 4), changes in ε_P mostly reflect changes in leakage. Consequently, cells seem to greatly reduce their leakage with increasing pH or decreasing CO_2 concentrations. Moreover, based on the large differences in ε_P, leakage appears to be highest in *C. lineatum* and lowest in *P. minimum*.

The efficiency of a CCM does not only depend on the kinetics of the carbon uptake systems but also on the loss of Ci via efflux. High leakage will increase the energetic costs of a CCM and/or decrease its capability to reach carbon saturation (Raven & Lucas 1985; Spalding & Portis 1985). In this respect, it is necessary to minimize the relative loss of Ci by leakage at low ambient CO_2 concentration, hereby increasing their overall CCM efficiency. At the same time, however, there is a greater potential for leakage owing to lower ambient CO_2 concentrations and higher K_M:$K_{1/2}$ ratio, both causing the outward CO_2 gradient to be higher. According to Fick's law, the CO_2 flux via a membrane is a function of the CO_2 concentration gradient and a permeability coefficient. Higher apparent resistance for CO_2 diffusion could be caused by changes in membrane properties, but it may also point to a more localized Ci accumulation in the chloroplast and to changes of the pyrenoid. Species-specific differences in leakage, as deduced from ε_P values, may reflect lower CCM efficiency in *C. lineatum* relative to *P. minimum* and *H. triquetra*.

We also estimated leakage in the Ci-flux assays from the CO_2 efflux observed directly upon darkening (Badger *et al.* 1994). The calculation is based on the assumption that leakage occurs mainly by diffusion of CO_2 and 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. This approach may underestimate the real Ci efflux due to re-fixation of CO_2 by internal ribulose-bisphosphate in the dark and a slow response time of the MIMS. Moreover, microalgae release relatively small amounts of CO_2 in the dark, as they build up only small internal Ci pools. Considering these uncertainties, absolute values for leakage should be treated with caution. In the comparison we also included *C. lineatum* because only relative numbers were used here. Cellular leakage differed between species (Fig. 8), being highest in *C. lineatum* and lowest in *H. triquetra*. These findings are consistent with the species-specific differences in ε_P.

CONCLUSIONS

Much of the effort in investigating carbon acquisition in marine phytoplankton has focused on diatoms and coccolithophores. Limited studies have focused on the experimentally less tractable dinoflagellates (Giordano *et al.* 2005). These few studies suggest that photosynthesis and growth in dinoflagellates may be CO_2 limited in the marine environment and that they probably are restricted to CO_2-enriched micro-environments (Colman *et al.* 2002; Dason *et al.* 2004). The data of our investigation do not support this view. All three dinoflagellates predominantly use HCO_3^- as their Ci source. For the two species that could be investigated using Ci-flux measurements, high affinities for Ci were found. This suggests that these dinoflagellates are not limited by Ci in open waters, which are characterized by only small changes in DIC and rather moderate changes in pH. In view of the ongoing increase in atmospheric pCO_2, concomitant changes in the carbonate chemistry will most likely not directly affect the rate of carbon fixation in these species.

However, could Ci be limiting for dinoflagellates in more productive coastal waters? In these environments, pH may become elevated and reach values above 9, and in some cases even up to 9.75, during red tides (Hansen 2002). Under such conditions, DIC will not only be significantly reduced, but a large part of the Ci pool will be in the form of CO_3^{2-}, which may be not directly accessible for the algae. It is known that the sensitivity of marine dinoflagellates to high pH differs among species. In pH-drift experiments, some dinoflagellates can grow until pH reaches 10.3, while the growth of others stops already at pH 8.3–8.4 (e.g. Hansen 2002). The species (and clones) used in the present study almost display the same magnitude of differences in their tolerance to high pH. *H. triquetra* and *P. minimum* will grow until pH reaches 9.4 and 9.6, respectively, while *C. lineatum* will stop growing at pH of 8.7. The three species are common in temperate-tropical coastal waters, where they often form red tides. However, *C. lineatum* forms blooms in more open coastal waters (e.g. the North Sea or the Kattegat/Skagerrak), while the two other species form dense blooms in highly eutrophic fjords (Fenchel *et al.* 1995; Lindholm & Nummelin 1999). Mixed growth experiments in the laboratory with the three species have shown that the differences in their sensitivity to pH are large enough to cause a succession of species so that the most pH tolerant species eventually out-competes the others (Hansen 2002).

The results presented here suggest that both *H. triquetra* and *P. minimum* are able to maintain high rates of carbon fixation at elevated pH by increasing their affinities for their carbon source HCO_3^-. Our findings are consistent with the observation that some HCO_3^- transporters are CO_2 induced (Omata *et al.* 1999). Such a strong response to pH or CO_2 concentrations may be somewhat surprising for an 'HCO_3^--user'. It should be considered, however, that at high pH not only the availability of CO_2, but also the concentration of HCO_3^-, decreases significantly. We also found evidence that the pH/CO_2-dependent responses in dinoflagellates, and possibly in other phytoplankton, may reflect their susceptibility to leakage; in other words, their way to minimize Ci losses and, hence, save energy. Future investigations should pay more attention to this phenomenon and explore the mechanisms behind reduced leakage in other algae to judge the significance of this process.

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