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 triqueta and Ceratium lineatum. In vivo activities of extracellular and intracellular carbonic anhydrase (CA), photosynthetic O₂ evolution, CO₂ and HCO₃⁻ 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 ¹⁴C-dis-equilibrium 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 (Kₒ) for photosynthetic O₂ 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 CO₂ concentration mechanism (CCM) in these dinoflagellates. Rates of CO₂ uptake were comparably low and could not support the observed rates of photosynthesis. Consequently, rates of HCO₃⁻ uptake were high in the investigated species, contributing more than 80% of the photosynthetic carbon fixation. The affinity for HCO₃⁻ and maximum uptake rates increased under higher pH. The strong preference for HCO₃⁻ was also confirmed by the ¹³C-dis-equilibrium technique. Modes of carbon acquisition were consistent with the ¹³C-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 triqueta; Prorocentrum minimum; ¹³C-fractionation; CO₂ concentrating mechanism; CO₂ uptake; HCO₃⁻ 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 CO₂ for carbon fixation. This highly conserved enzyme is characterized by a low affinity for its substrate CO₂, slow maximum specific turnover rate and susceptibility to a competing reaction with O₂. The latter reaction initiates the process of photorespiration, which further lowers the rate of carbon fixation.

Under present atmospheric conditions (i.e. low CO₂ and high O₂ levels), the catalytic inefficiency of Rubisco imposes restrictions on the carbon assimilation of all photoautotrophs. This is particularly true for algae as CO₂ availability in water is further reduced, owing to slow CO₂ diffusion rate, slow conversion rate between HCO₃⁻ and CO₂ and low CO₂ concentrations under alkaline conditions. To avoid carbon limitation in photosynthesis, algae developed mechanisms to enhance intracellular CO₂ concentration at the site of carboxylation. These CO₂ concentrating mechanisms (CCMs) involve active uptake of CO₂ and/or HCO₃⁻, as well as of the enzyme carbonic anhydrase (CA), which accelerates the otherwise slow conversion rate between HCO₃⁻ and CO₂. 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. Burkhart et al. 2001; Beadall & 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 CO₂-sensitive in their photosynthesis, whereas other species are rate-saturated even under low ambient CO₂ 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
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 ($S_{\text{co}}$), 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 & Merritt 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 Cl 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 Cl pool will be in the form of CO$_2$–, 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 Cl 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 ($\varepsilon_p$) 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 Cl 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 Cl 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 ¹⁸O-depletion of doubly labelled ¹³C¹⁸O₂ in water caused by several CO₂ and HCO₃⁻ 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 CO₂ isotopomers ¹³C¹⁸O₂ (m/z = 49), ¹³C₁₆O¹₈O (m/z = 47) and ¹³C₁₆O¹⁸O (m/z = 45) were recorded continuously and calculated as:

\[
^{18}O\ log(\text{enrichment}) = \log \left( \frac{13C^{18}O_2 \times 100}{13CO_2} \right) = \log \left( \frac{49 \times 100}{45 + 47 + 49} \right) \tag{1}
\]

Measurements of eCA and iCA activities were performed in the CO₂-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. NaH¹³C¹₈O₃ was added to a final concentration of 1 mmol L⁻¹ and the uncatalysed rate of ¹⁸O 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 ¹⁸O-atom fraction after the addition of the sample (S₂) was compared to the non-catalysed decline (S₁) and normalized on a Chl a basis (Badger & Price 1989):

\[
U = \frac{(S_2 - S_1) \times 100}{S_1 \times \text{mg Chl a}} \tag{2}
\]

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 sulfonylamide (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 ¹⁸O exchange.

**Isotope-disequilibrium experiments**

The ¹⁴C-disequilibrium technique makes use of the transient isotopic disequilibrium upon an acidic ¹⁴C spike into cell suspension at high pH to determine whether CO₂ or HCO₃⁻ 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 ¹⁴C 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 mmol L⁻¹). After the injection of the ¹⁴C spike, 200 µL subsamples were withdrawn at short intervals and dispersed into 1.5 mL of HCl (6 N). To remove residual inorganic ¹⁴C 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 ¹⁴C was measured by standard liquid scintillation procedures. To correct for small residual inorganic ¹⁴C, blanks consisting of spike added to cell-free buffer were measured.

For quantitative interpretation of ¹⁴C-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 CO₂ and HCO₃⁻ uptake at any time and is given by (Elzenga et al. 2000):

\[
d(DPM_t)/dt = V_{CO_2} \times S_{A_{CO_2}} \times V_{HCO_3} \times S_{A_{HCO_3}} \tag{3}
\]
where \( \frac{d(DPM)}{dt} \) 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. \( SA_{CO_2} \) and \( SA_{HCO_3} \) 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):

\[
SA_{CO_2} = SA_{bic} + \Delta SA_{CO_2} \times e^{-at} \quad \text{and} \quad \Delta SA_{CO_2} = \frac{V(1-f)\times[\alpha_1 t + (\Delta SA_{CO_2} / SA_{bic}) \times (1-e^{-at})]}{\alpha_2} + V(f)\times[\alpha_2 t + (\Delta SA_{HCO_3} / SA_{bic}) \times (1-e^{-at})]/\alpha_2.
\]

The values of \( \Delta SA_{CO_2} / \Delta SA_{bic} \) and \( \Delta SA_{HCO_3} / \Delta SA_{bic} \) 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 activity 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 δ\(^{13}\)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):

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

Isotope fractionation during POC formation \( (\varepsilon_p) \) was calculated relative to the isotopic composition of CO\(_2\) in the medium (Freeman & Hayes 1992):

\[
\varepsilon_p = \frac{\delta^{13}C_{CO_2} - \delta^{13}C_{POC}}{1 + \delta^{13}C_{POC}}.
\]

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):

\[
\delta^{13}C_{HCO_3^-} = \frac{\delta^{13}C_{DIC} + (\varepsilon_p[CO_2] + \varepsilon_p[CO_3^-])}{(1 + \varepsilon_p \times 10^{-3})[CO_2] + [HCO_3^-]} + (1 + \varepsilon_p \times 10^{-3})[CO_3^-]
\]

\[
\delta^{13}C_{CO_2} = \delta^{13}C_{HCO_3^-}(1 + \varepsilon_p \times 10^{-3}) + \varepsilon_p.
\]
Temperature-dependent fractionation factors between CO$_2$ and HCO$_3^-$ ($\varepsilon_f$) as well as HCO$_3^-$ and CO$_2^+$ ($\varepsilon_f$) 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.

**14C-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$_2$ uptake of 18O-labelled 13CO$_2$ (m/z = 45) and the 18O log (enrichment) by cells of *Heterocapsa triquetra* (a) as well as *Ceratium lineatum* (b). Subsequent to the illumination of *H. triquetra* resulted in a faster uptake of 18O-labelled 13CO$_2$ (m/z = 49 and 47) and consequent efflux of unlabelled 13CO$_2$ (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 18O exchange in *C. lineatum*. In the latter, light-stimulation in the 18O 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.

**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).

<table>
<thead>
<tr>
<th>pH</th>
<th>eCA [U (mg Chl $a$)$^{-1}$]</th>
<th>iCA [A (mg Chl $a$)$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Prorocentrum minimum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>47 ± 15</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>9.1</td>
<td>199 ± 24</td>
<td>24 ± 1</td>
</tr>
<tr>
<td><em>Heterocapsa triquetra</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>13 ± 3</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>9.1</td>
<td>26 ± 10</td>
<td>4 ± 1</td>
</tr>
<tr>
<td><em>Ceratium lineatum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>25 ± 14</td>
<td>32 ± 8</td>
</tr>
<tr>
<td>8.5</td>
<td>34 ± 2</td>
<td>29 ± 1</td>
</tr>
</tbody>
</table>

CA, carbonic anhydrase.

depletion reached a steady state. When the light was turned off, photosynthetic O₂ production ceased immediately and was replaced by respiratory O₂ consumption. The rapid CO₂ increase was caused by both re-equilibration and CO₂ efflux. Based on such changes in O₂ and CO₂ concentration rates of net photosynthesis, CO₂ and HCO₃⁻ uptake were calculated and expressed as a function of CO₂ and/or HCO₃⁻ 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 (*K₅/₂*) and maximum rates (*V₅ₐₓ*) 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 *V₅ₐₓ* of photosynthesis, *P. minimum* and *H. triquetra* were quite similar and the acclimation pH had

<table>
<thead>
<tr>
<th>pH</th>
<th>Fraction HCO₃⁻ DBS</th>
<th>Fraction HCO₃⁻ Control</th>
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</thead>
<tbody>
<tr>
<td><em>Prorocentrum minimum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>0.83 ± 0.022</td>
<td>0.81 ± 0.024</td>
</tr>
<tr>
<td>9.1</td>
<td>0.94</td>
<td>0.92</td>
</tr>
<tr>
<td><em>Heterocapsa triquetra</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>0.85 ± 0.002</td>
<td>0.92 ± 0.016</td>
</tr>
<tr>
<td>9.1</td>
<td>0.86 ± 0.002</td>
<td>0.92 ± 0.012</td>
</tr>
<tr>
<td><em>Ceratium lineatum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>0.85 ± 0.017</td>
<td>0.84 ± 0.028</td>
</tr>
<tr>
<td>8.5</td>
<td>0.85 ± 0.005</td>
<td>0.85 ± 0.021</td>
</tr>
</tbody>
</table>

DBS, dextran-bound sulfonamide.

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 $K_{1/2}$ ($\text{HCO}_3^-$) 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, $K_{1/2}$ ($\text{CO}_2$) for photosynthesis were much lower than could be expected from diffusive $\text{CO}_2$ uptake alone. With values between 0.5 and 8.1 $\mu\text{mol L}^{-1} \text{CO}_2$, 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 $\mu\text{mol L}^{-1} \text{CO}_2$ 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 $\mu\text{mol L}^{-1}$ HCO$_3^-$ for *P. minimum* and from 426 to 26 $\mu\text{mol L}^{-1}$ 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.

**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$_{Ci}$), allowing us to calculate $\delta^{13}$C fractionation of the cells. Fractionation values ($\varepsilon$) 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

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**Figure 7.** Maximum rates ($V_{\text{max}}$) 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 were calculated from a Michaelis–Menten fit to the combined data of several independent measurements. Error bars denote ± SD ($n = 3$).

**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.
acclimation caused εF to decrease by 5 to 10‰, even yielding negative εF values (−1.1‰) in P. minimum. Such low εF values were consistent with predominant HCO$_3^-$ use. Observed εF 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, CO$_2$ limitation may be more severe during algal blooms when the pH is high. In this study, several aspects of CO$_2$ acquisition in three bloom-forming dinoflagellates were investigated by applying different independent methods, such as MIMS techniques for CA activity and CO$_2$ fluxes, as well as the 14C-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 14C-disequilibrium technique, which yielded similar rates of 14C incorporation in DBS-treated, as well as in nontreated, cells. Although some eCA activity could be detected by monitoring 18O 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 *Skel- etonema costatum* or the prymnesiophyte *Phaeocystis globo- sosa*, 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 18O 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 errors.

![Figure 8](image_url) **Figure 8.** Leakage (CO$_2$ efflux : Ci uptake) as a function of the CO$_2$ concentration in the assay, obtained from cells of *Prorocentrum minimum*, *Heterocapsa triqueta* and *Ceratium lineatum acclimated to high pH.

<table>
<thead>
<tr>
<th>pH</th>
<th>εP (%)</th>
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<tbody>
<tr>
<td>8.0</td>
<td>9.0</td>
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<tr>
<td>9.1</td>
<td>10.0</td>
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![Figure 9](image_url) **Figure 9.** Isotope fractionation (εF) as a function of pH in the acclimation, calculated from the 14C$_{CO2}$ and 14C$_{Proc}$ 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 (Sülttemeyer 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 18O-depletion but also on the diffusive influx of doubly labelled CO₂, 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 CO₂ supply. Our data does confirm such pH dependence in iCA activity only for *P. minimum*.

Using the 18O-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 18O-labelled CO₂ and HCO₃⁻ into the cells to the active site of iCA, increased 18O loss and subsequent efflux of 18O-unlabelled CO₂ (Badger & Price 1989; Palmqvist et al. 1994). Such a net CO₂ efflux from photosynthesizing cells result from the accumulation of CO₂ inside the cell relative to ambient CO₂ concentration. As shown in Fig. 2, illumination of *H. triqueta* resulted in an uptake of 18O-18O₂ (m/z = 49) and an efflux of 18O-18O₂ (m/z = 45), that is, a light-dependent decrease in log(enrichment).

Similar 18O 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 18O 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 CO₂, causing the enrichment to rise rather than to fall (see Badger et al. 1998).

### Photosynthetic O₂ 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. triqueta* remained unaltered over the duration of the assay. In fact, *Vₘₐₓ* 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 O₂ evolution as a function of CO₂ concentration in the assays provides important information on the carbon acquisition of microalgae. Lower apparent *K₁/₂* (CO₂) than *Kₘ* (CO₂) 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, *K₁/₂* (CO₂) 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. triqueta* both responded to high pH by increasing the apparent affinities for Ci. In *P. minimum*, *K₁/₂* (CO₂) of photosynthesis decreased from 3.2 to 1.6 μmol L⁻¹ with increasing pH in the acclimation. In *H. triqueta*, acclimation to high pH had a stronger effect on the up-regulation of the CCM, causing *K₁/₂* (CO₂) 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 *K₁/₂* (CO₂) 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 *K₁/₂* (CO₂) 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 *K₁/₂* (CO₂) of photosynthesis relative to the *Kₘ* (CO₂) of Rubisco suggest high accumulation of CO₂ at the site of Rubisco. The *Kₘ*:*K₁/₂* ratio approximates for such an internal CO₂ enrichment relative to ambient CO₂ 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 *S₄ₙ* values obtained for *A. carterae* (Whitney & Andrews 1998). Assuming a *Kₘ* of 60 μmol L⁻¹ (Leggat et al. 1999), the *Kₘ*:*K₁/₂* ratio indicate a CO₂ accumulation of about 19- to 37-fold in *P. minimum*. In *H. triqueta*, *K₁/₂* (CO₂) values could theoretically be achieved by a CO₂ 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. carterae*, 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 \( K_{1/2} \) ratios, which indicate rather high CO2 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 CO2 accumulation will require full kinetic characterization of dinoflagellate Rubisco.

**Carbon source and uptake kinetics**

Although CO2 dependence in O2 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 CO2 and HCO3 uptake in microalgae. In this study, estimates of net CO2 and HCO3 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 CO2 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 CO2 to the overall carbon fixation, \( K_{1/2} \) values could not be calculated for *P. minimum*. In *H. triqueta* apparent affinities for CO2 increased with pH. Both species caused the CO2 concentration to decrease below disequilibrium concentrations in the light, which has been confirmed by the addition of bovine CA (data not shown). Because CO2 uptake could not support the observed rates of photosynthesis, most of the Ci was taken up as HCO3. In both species, HCO3 uptake contributed more than 80% of the total carbon fixation. Apparent affinities for HCO3 were generally higher for *P. minimum* than for *H. triqueta*. Acclimation to high pH caused a strong increase in apparent affinities for HCO3, indicated by the generally lower \( K_{1/2} \) values.

In a second approach, we applied the 14C-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 CO2 or HCO3 uptake can be calculated by this approach. The proportion of HCO3 relative to carbon fixation was high in all three species, making up between about 83 and 95% (Table 2). Comparing the relative contribution of HCO3 obtained by this approach with those obtained by Ci-flux measurements (MIMS), we get similar results (Fig. 4). This assures HCO3 as the dominant carbon source taken up.

Our findings contradict with a recent study on free-living dinoflagellates, which found no evidence for HCO3 use in *A. carterae* and *H. oceanica* (Dason et al. 2004). Their conclusion is mainly based on rather low rates of photosynthetic O2 evolution in comparison to the spontaneous rate of CO2 formation. We argue that while rates of photosynthesis higher than the spontaneous CO2 delivery from the HCO3 pool may indicate HCO3 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 CO2 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 CO2 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, 14C-incorporation rates with and without DBS in Fig. 3).

**Fractionation and leakage**

Photosynthetic carbon fixation discriminates against the heavier 13CO2 causing the isotopic composition of organic material (δ13CPOC) to be depleted in 13C, compared with the Ci source. Most of this fractionation (\( \varepsilon_p \)) is driven by the discrimination of 13C by Rubisco (\( \varepsilon_f \)), here assumed to be about 30‰ (Raven & Johnston 1991). While \( \varepsilon_f \) sets the uppermost values for \( \varepsilon_p \), variations are principally determined by Ci leakage (L) and the carbon source taken up (Sharkey & Berry 1985):

\[
\varepsilon_p = a \times \varepsilon_f + L \times \varepsilon_c.
\]  

(11)

\( \varepsilon_f \) represents the equilibrium discrimination between the carbon sources CO2 and HCO3 (approx −10‰) and \( a \) is the fractional contribution of HCO3 to total Ci uptake. Because HCO3 is enriched in 13C relative to CO2, an increasing proportion of HCO3 uptake decreases the apparent isotope fractionation \( \varepsilon_p \), which is defined relative to CO2 as the carbon source. If there is no change in the Ci source, \( \varepsilon_p \) 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 \( \varepsilon_p \) values allow precluding one carbon source. If \( \varepsilon_p \) is lower than 0‰, CO2 can be excluded as the only carbon source and \( \varepsilon_p \) values higher than 20‰ rule out HCO3 as the only carbon source. \( \varepsilon_c \) values in our experiments (Fig. 9) are within this range, which is consistent with predominant HCO3 use. The negative \( \varepsilon_p \) values of −1.1‰ in *P. minimum* even precluded CO2 as the only carbon source. Berman-Frank et al. (1998) observed high δ13CPOC 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 εp to drop by 5–10%. As HCO3− is the predominant carbon source under both pH acclimations (Fig. 4), changes in εp mostly reflect changes in leakage. Consequently, cells seem to greatly reduce their leakage with increasing pH or decreasing CO2 concentrations. Moreover, based on the large differences in εp, 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 CO2 concentration, hereby increasing their overall CCM efficiency. At the same time, however, there is a greater potential for leakage owing to lower ambient CO2 concentrations and higher \( K_{\text{a}}/K_{\text{s}} \) ratio, both causing the outward CO2 gradient to be higher. According to Fick’s law, the CO2 flux via a membrane is a function of the CO2 concentration gradient and a permeability coefficient. Higher apparent resistance for CO2 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 εp 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 CO2 efflux observed directly upon darkening (Badger et al. 1994). The calculation is based on the assumption that leakage occurs mainly by diffusion of CO2 and that the rate of diffusive CO2 efflux in the light is well represented by the rate of CO2 efflux during the first seconds of the subsequent dark phase. This approach may underestimate the real Ci efflux due to re-fixation of CO2 by internal ribulose-bisphosphate in the dark and a slow response time of the MIMS. Moreover, microalgae release relatively small amounts of CO2 in the dark, as they build up only small internal Ci pools. Considering these uncertainties, absolute values for loss of Ci by leakage at low ambient CO2 concentration, \( \epsilon \), leak- age appears to be highest in C. lineatum and lowest in P. minimum.

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 CO2 limited in the marine environment and that they probably are restricted to CO2−-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 HCO3− 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 pCO2, 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 CO3−2, 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 HCO3−. Our findings are consistent with the observation that some HCO3− transporters are CO2 induced (Omata et al. 1999). Such a strong response to pH or CO2 concentrations may be somewhat surprising for an ‘HCO3−-user’. It should be considered, however, that at high pH not only the availability of CO2, but also the concentration of HCO3− decreases significantly. We also found evidence that the pH/CO2-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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