INORGANIC CARBON ACQUISITION OF MARINE PHYTOPLANKTON WITH EMPHASIS ON SELECTED DIATOM SPECIES

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This study investigates the inorganic carbon (C_i) acquisition in marine phytoplankton with emphasis on selected diatom species and the role of calcification in the photosynthetic carbon acquisition of the coccolithophore *Emiliania huxleyi*. Observations from laboratory and field experiments delivered new insights into the efficiency and regulation of carbon concentrating mechanisms (CCM) in diatoms in response to varying seawater carbonate chemistry. The main motivation for the experiments was to examine the role of carbon acquisition in phytoplankton ecology and to assess the potential effects of elevated CO_2 on marine diatoms.

The CCM of the four bloom-forming diatom species, *Pseudo-nitzschia multiseries*, *Eucampia zodiacus*, *Skeletonema costatum*, *Thalassionema nitzschioides* and the three non-bloom-forming species *Thalassiosira pseudonana, Nitzschia navis-varingica*, and *Stellarima stellaris* has been characterised in response to CO_2/pH-induced changes in seawater carbonate chemistry by means of membrane inlet mass spectrometry (MIMS). With the exception of *T. pseudonana* and *N. navis-varingica*, extracellular carbonic anhydrase (eCA) activities increased with decreasing CO_2 supply in all investigated diatoms. Half-saturation concentrations ($K_{1/2}$) for photosynthetic O_2 evolution in the examined diatoms were generally significantly lower than K_M (CO_2) of Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) in diatoms, indicating the operation of a highly efficient CCM. When exposed to low carbon supply, $K_{1/2}$ values for photosynthetic O_2 evolution generally decreased in the investigated diatom species, demonstrating CO_2-dependent regulation of CCMs in diatoms.

In terms of carbon source, all species took up both CO_2 and HCO_3^-. Despite these similarities, C_i uptake kinetics differed strongly among species. With respect to the preferred C_i source, the contribution of HCO_3^- to net fixation was more than 80% in *S. stellaris*, *E. zodiacus* and *S. costatum* ('HCO_3^- users'), while it was about 50% in *P. multiseries*, *T. nitzschioides* and *T. pseudonana* and only ~30% in *N. navis-varingica* ('CO_2 user'). The comparison of bloom-forming and non-bloom-forming diatoms revealed that diatoms generally have a high plasticity in terms of the preferred carbon source and the degree of regulation in C_i uptake enables this group to adjust the rates of C_i uptake to the actual C_i availability.

In the current study a strong correlation between high eCA activity and predominant HCO_3^- uptake as well as lacking eCA activity in cells predominantly taking up CO_2 was found. These observations call into question the general notion that eCA functions to provide CO_2 to the uptake systems at high pH. To explain this apparent contradiction, a

novel role for eCA acting as C_i-recycling mechanism is proposed. According to this, the presence of eCA in 'HCO_3^- users' such as *S. stellaris* serves to recycle CO_2 by converting the CO_2 leaking out of the cell to HCO_3^- that is subsequently taken up by HCO_3^- transporters. For 'CO_2 users' such as *N. navis-varingica*, the absence of eCA may cause the CO_2 leaking out of the cell to be prevented from fast conversion to HCO_3^- and a disequilibrium at the cell surface persists. Elevated CO_2 in turn increases the probability that the CO_2 is transported back into the cell via CO_2 transport systems. It is concluded that the presence or absence of eCA allows for a more efficient C_i-recycling in 'HCO_3^-' and 'CO_2 users', respectively.

It is a common notion that 'HCO_3^- users' have a competitive advantage over 'CO_2 users', especially under high pH, and thus would be less sensitive to changes in carbonate chemistry. However, for 'HCO_3^- users' like *S. stellaris*, growth was already affected above pH 8.8. In contrast, 'CO_2 users' such as *N. navis-varingica* grew up to pH values of 10. Furthermore, the 'HCO_3^- users' *S. stellaris*, *S. costatum* and *E. zodiacus* exhibited much higher leakage (70% CO_2 efflux to the total C_i uptake) than the 'CO_2 user' *N. navis-varingica* (20%). Additionally, the apparent affinities for C_i were high in all investigated diatom species irrespective of the preferred carbon source. It can thus be concluded that 'HCO_3^- users' are as sensitive as or even more sensitive than 'CO_2 users' with regard to their pH/CO_2-dependence in photosynthesis and growth. Thus, 'HCO_3^- users' are not necessarily less sensitive to changes in carbonate chemistry than 'CO_2 users', an observation that might be related to differences in leakage.

During bloom situations phytoplankton have to cope with decreasing CO_2 concentrations owing to high photosynthetic activity. While for diatoms a C_4-like photosynthetic pathway has been suggested to operate under these conditions, for coccolithophores calcification would provide an additional source of CO_2 under low CO_2 availability. To investigate whether the three bloom-forming diatoms *E. zodiacus*, *S. costatum*, *T. nitzschioides* and the non-bloom-forming *T. pseudonana* operate a C_4-like pathway, activities of RubisCO and phosphoenolpyruvate carboxylase (PEPC) were measured in cells acclimated to different CO_2 levels. Independent of the CO_2 supply, PEPC activities were significantly lower than those of RubisCO, averaging generally less than 3% of the activity of RubisCO. The absence of C_4 metabolism in the investigated species is further indicated by relatively high carbon isotope fractionation values (ε_p), which varied between 10 and 16‰.

To examine the relationship between photosynthesis and calcification in coccolithophores, cells of a calcifying strain of *E. huxleyi* were grown at four calcium (Ca^{2+}) concentrations leading to different degrees of calcification in the same strain. By measuring the responses in photosynthesis and calcification as well as applying MIMS techniques, it

was demonstrated that photosynthesis remained constant when calcification ceased. No differences in ε_p were observed under different Ca^{2+} concentrations. The ratio of photosynthetic HCO_3^- uptake relative to net fixation did not differ in calcifying and non-calcifying cells. Based on these results it is concluded that calcification is not involved in photosynthetic carbon acquisition in the coccolithophore *E. huxleyi*.

Field studies with natural phytoplankton assemblages in the Ross Sea (Antarctica) demonstrated that C_i uptake systems of marine phytoplankton are regulated by the CO_2 differences *in situ*. Shipboard incubation experiments revealed that C_i uptake, primary productivity and diatom species composition are sensitive to CO_2. Results of the $^{14}C_i$ kinetic assay showed that apparent C_i affinities decreased by about 2-fold under high pCO_2. By means of the ^{14}C disequilibrium technique, it was determined that the fraction of HCO_3^- relative to net fixation was high, generally 90%, while maximum C_i transport rates decreased ~2-fold under high CO_2 levels, indicating lower C_i transport capacity under the latter conditions. The decrease in the maximum C_i transport capacity and the lower apparent C_i affinities, both reflecting a down-regulation of the CCM activity, may explain the higher primary productivity and growth observed in phytoplankton assemblages under elevated CO_2. Additionally, high pCO_2 levels triggered the growth of large chain-forming diatom species. This is consistent with laboratory results indicating that large diatoms such as *S. stellaris* are likely to benefit most from the projected increase in CO_2. The findings of this study demonstrate that the projected changes in seawater carbonate chemistry are likely to induce a species shift within diatoms, which could translate into enhanced vertical fluxes of particulate organic carbon from surface waters to the deep ocean representing a negative feedback on increased atmospheric CO_2.

2 ZUSAMMEN-

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Diese Arbeit untersucht den anorganischen Kohlenstofferwerb von marinem Phytoplankton mit Schwerpunkt auf ausgewählten Diatomeenarten und die Rolle der Kalzifizierung im photosynthetischen Kohlenstofferwerb der Coccolithophoride *Emiliania huxleyi*. Beobachtungen aus Labor- und Feldexperimenten lieferten neue Einblicke in die Effizienz und die Regulation von Kohlenstoffkonzentrierungsmechanismen (CCM) in Diatomeen in Hinblick auf Änderungen in der Karbonatchemie des Meerwassers. Neben der Klärung der Rolle von Komponenten des CCMs in der Phytoplanktonökologie galt es mögliche Folgen von erhöhtem CO_2 auf die Gruppe der Diatomeen abzuschätzen.

Mit Hilfe von Membran Einlass Massenspektrometrie (MIMS) wurde der CCM in den vier blütenbildenden Diatomeenarten *Pseudo-nitzschia multiseries*, *Eucampia zodiacus*, *Skeletonema costatum*, *Thalassionema nitzschioides* und den drei nicht-blütenbildenden Arten *Thalassiosira pseudonana, Nitzschia navis-varingica*, and *Stellarima stellaris* in Hinblick auf die durch CO_2/pH hervorgerufenen Änderungen in der Karbonatchemie des Meerwassers charakterisiert. Mit Ausnahme von *T. pseudonana* and *N. navis-varingica* ist die Aktivität der extrazellulären Carboanhydrase (eCA) mit abnehmendem CO_2 Angebot in allen untersuchten Diatomeen gestiegen. Die Halbsättigungskonzentrationen ($K_{1/2}$) für die O_2-Entwicklung aller untersuchten Diatomeen waren generell signifikant niedriger als die Halbsättigungskonstanten (K_M) für CO_2 von Ribulose-1,5-Biphosphate Carboxylase/Oxygenase (RubisCO), was auf einen hoch effizienten CCM hindeutet. Bei niedrigem CO_2 Angebot sind die $K_{1/2}$-Werte für die photosynthetische O_2-Entwicklung in den untersuchten Diatomeenarten generell gesunken, was wiederum eine CO_2 abhängige Regulation des CCM in Diatomeen andeutet.

Mit bezug auf die Kohlenstoffquelle kann gesagt werden, dass alle Arten CO_2 und HCO_3^- aufnahmen. Trotz dieser Gemeinsamkeiten zeigten die Arten große Unterschiede in den Kinetiken für die Aufnahme von anorganischem Kohlenstoff. Hinsichtlich der bevorzugten anorganischen Kohlenstoffspezies betrug der HCO_3^- Anteil zur Nettofixierung mehr als 80% in *S. stellaris*, *E. zodiacus* and *S. costatum* ('HCO_3^- Nutzer'), während er ungefähr etwa 50% in *P. multiseries*, *T. nitzschioides* and *T. pseudonana* und nur ~30% in *N. navis-varingica* ('CO_2 Nutzer') betrug. Der Vergleich von blütenbildenden mit nicht-blütenbildenden Diatomeen hat gezeigt, dass Diatomeen generell eine sehr hohe Diversität in Hinblick auf die bevorzugte Kohlenstoffquelle haben, sowie ihre anorganischen

Kohlenstoffaufnahmeraten an das aktuelle anorganische Kohlenstoffvorkommen anpassen können.

In der vorliegenden Arbeit wurde eine starke Korrelation zwischen hoher eCA Aktivität und überwiegender HCO_3^- Aufnahme als auch dem Fehlen von eCA Aktivität und überwiegender CO_2 Aufnahme gefunden. Diese Beobachtungen stellen die zurzeit allgemein gültige Annahme in Frage, dass eCA dazu dient, CO_2 den Aufnahmesystemen bei hohem pH bereitzustellen. Um diesen offensichtlichen Widerspruch zu erklären, wird eine neue Rolle der eCA als anorganischer Kohlenstoff-Recyclingsmechanismus vorgeschlagen. Hiernach dient das Vorkommen von eCA in ‚HCO_3^- Nutzern' wie *S. stellaris* dazu, CO_2 zu recyceln, indem das CO_2, das aus der Zelle entweicht, zu HCO_3^- umgewandelt wird, welches wiederum dann von den HCO_3^- Transportern aufgenommen wird. Für ‚CO_2 Nutzer' wie *N. navis-varingica* führt gerade die Abwesenheit von eCA dazu, das das CO_2, das aus der Zelle entweicht, nicht zu HCO_3^- umgewandelt wird, und ein Ungleichgewicht auf der Zelloberfläche entsteht. Erhöhtes CO_2 wiederum erhöht die Wahrscheinlichkeit, dass CO_2 mit Hilfe von CO_2 Transporter in die Zelle zurückgebracht wird. Es wird geschlussfolgert, dass die An- oder die Abwesenheit von eCA einem effizienten Recycling von anorganischem Kohlenstoff sowohl in ‚HCO_3^--' als auch in ‚CO_2 Nutzern' dient.

Allgemein geht man davon aus, dass 'HCO_3^- Nutzer' besonders bei hohem pH einen kompetitiven Vorteil gegenüber ‚CO_2 Nutzern' haben und dementsprechend weniger sensitiv gegenüber Veränderungen in der Karbonatchemie sind. Das Wachstum von ‚HCO_3^- Nutzern' wie *S. stellaris* war jedoch schon bei einem pH von mehr als 8.8 eingeschränkt. Im Gegensatz hierzu konnten ‚CO_2 Nutzer' wie *N. navis-varingica* bis zu einem pH von 10 wachsen. Des Weiteren zeigten die ‚HCO_3^- Nutzer' *S. stellaris*, *S. costatum* und *E. zodiacus* sehr viel höheres Leakage (70% CO_2 Efflux gegenüber der Gesamtkohlenstoffaufnahme) als der ‚CO_2 Nutzer' *N. navis-varingica* (20%). Auch die apparenten Affinitäten für anorganischen Kohlenstoff waren in allen untersuchten Diatomeenarten unabhängig von der bevorzugten Kohlenstoffquelle. Daraus kann gefolgert werden, dass ‚HCO_3^- Nutzer' genauso sensitiv oder sogar sensitiver sind als ‚CO_2 Nutzer' in Bezug auf ihre pH/CO_2-Abhängigkeit der Photosynthese und des Wachstums. Folglich sind ‚HCO_3^- Nutzer' nicht zwangsläufig weniger sensitiv als ‚CO_2 Nutzer' gegenüber Veränderungen der Karbonatchemie, eine Beobachtung, die auf Unterschiede im Leakage zurückzuführen sein kann.

Während einer Blütensituation müssen Phytoplankter mit abnehmenden CO_2 Konzentrationen bedingt durch hohe photosynthetische Aktivität zurechtkommen. Während für Diatomeen vorgeschlagen wurde, dass sie unter diesen Bedingungen auf einen C_4-ähnlichen Photosynthese Stoffwechselweg zurückgreifen, könnte für Coccolithophoriden

die Kalzifizierung eine zusätzliche CO_2-Quelle unter niedrigem CO_2 Angebot darstellen. Um zu testen, ob die drei blütenbildenden Diatomeenarten *E. zodiacus*, *S. costatum*, *T. nitzschioides* und die nicht-blütenbildende *T. pseudonana* einen C_4-Stoffwechselweg haben, wurden die Aktivitäten von RubisCO und Phosphoenolpyruvat Carboxylase (PEPC) in Zellen bestimmt, die an unterschiedliche CO_2-Partialdrücke (pCO_2) akklimiert wurden. Unabhängig vom CO_2 Angebot waren die Aktivitäten von PEPC signifikant geringer gegenüber denen von RubisCO, im Durchschnitt betrugen sie weniger als 3% der Aktivität von RubisCO. Die Abwesenheit des C_4 Stoffwechsels wurde weiterhin durch relative hohe Kohlenstoffisotopie Werte (ε_p) zwischen 10 und 16‰ angezeigt.

Um die Beziehung zwischen Photosynthese und Kalzifizierung in Coccolithophoriden zu untersuchen, wurde Zellen eines kalzifizierenden Strains von *Emiliania huxleyi* unter vier Kalziumkonzentrationen angezogen, die zu unterschiedlichen Kalzifizierungsgraden im selben Strain geführt haben. Durch Photosynthese-, Kalzifizierungs- und MIMS Messungen konnte gezeigt werden, dass die Photosynthese konstant blieb, während die Kalzifizierung abnahm. Es wurden keine Unterschiede in ε_p unter den unterschiedliche Ca^{2+}-Konzentrationen beobachtet. Das Verhältnis von photosynthetischer HCO_3^- Aufnahme relativ zur Nettofixierung war in kalzifierenden und nichtkalzifizierenden Zellen gleich. Auf Grundlage dieser Ergebnisse wird geschlussfolgert, dass die Kalzifizierung nicht an der photosynthetischen Kohlenstoffaufnahme der Coccolithophoride *E. huxleyi* beteiligt ist.

Feldstudien mit natürlichen Phytoplanktongesellschaften des Rossmeeres (Antarktis) haben gezeigt, dass die anorganischen Kohlenstoffaufnahmesysteme von marinem Phytoplankton von den CO_2 Unterschieden *in situ* reguliert sind. Schiffinkubaktionsexperimente haben gezeigt, dass die anorganische Kohlenstoffaufnahme, die Primärproduktivität und die Diatomeenzusammen-setzung sensitiv gegenüber unterschiedlichen CO_2-Patialdrücken sind. Die Ergebnisse des $^{14}C_i$ Kinetik Assays haben gezeigt, dass die apparenten Affinitäten für anorganischen Kohlenstoff um das Zweifache unter hohen CO_2 Partialdrücken abnahmen. Mit Hilfe der ^{14}C Disequlibrium Technik wurde festgestellt, dass das Verhältnis von HCO_3^- Aufnahme zur Nettofixierung generell hoch war, mit typischerweise 90%, während die maximalen Transportraten des anorganischen Kohlenstoffs um das Zweifache unter hohen CO_2-Partialdrücken abnahm, was wiederum eine geringere Transportkapazität für anorganischen Kohlenstoff unter den genannten Bedingungen anzeigt. Die Abnahme in der maximalen Transportkapazität für anorganischen Kohlenstoff und die geringeren apparenten Affinitäten für anorganischen Kohlenstoff, die beide eine Erniedrigung der CCM-Aktivität anzeigen, könnten die höhere Primärproduktivität und das Wachstum in den beobachtenden Phytoplanktongesellschaften

unter erhöhtem CO_2 erklären. Des Weiteren führten hohe CO_2 Partialdrücke zu einem Anstieg des Wachstums von großen und kettenbildenden Diatomeenarten. Dies stimmt überein mit Laborergebnissen, die anzeigen, dass großen Diatomeen wie *S. stellaris* am meisten von dem zukünftigen CO_2 Anstieg profitieren werden. Die Ergebnisse dieser Arbeit verdeutlichen, dass die zukünftigen Änderungen in der Karbonatchemie des Meerwassers eine Verschiebung der Artenzusammensetzung innerhalb der Diatomeen bewirken können, die zu verstärkten vertikalen Flüssen von partikulärem organischem Material vom Oberflächenwasser in die Tiefe des Ozeans führen können und somit ein negatives Feedback auf den Anstieg im atmosphärische CO_2 darstellen können.

3 General Introduction

3 GENERAL INTRODUCTION

3.1 ECOLOGICAL ROLE OF DIATOMS AND COCCOLITHOPHORES

Marine phytoplankton comprise ~30000 species of marine photoautotrophs (Falkowski & Raven 1997). These photosynthetic organisms are responsible for more than 45% of the Earth's primary production (Field et al. 1998). Marine phytoplankton are a key player in driving the biological carbon pumps. Two types of biological pump can be distinguished, the organic carbon pump and the carbonate pump. The organic carbon pump is driven by photosynthetic carbon fixation of phytoplankton while the formation of particulate inorganic carbon affects the carbonate pump. Diatoms solely drive the organic carbon pump, but contribute up to 40% of the ocean's primary production (Nelson et al 1995) and thus play a major role in the downward transport of particulate organic carbon from surface waters to the deep ocean (Buesseler 1998). Diatoms are unique among phytoplankton in that they require silicon, in the form of silicic acid for growth and the production of their frustules. They play a key role in marine silicon cycling (Tréguer et al. 1995) and are mainly involved in the biogeochemical cycles of nitrogen, phosphorus, and trace metals like iron. More than 5000 extant marine diatom species have been described (Falkowski et al. 2004), the evolutionary roots of which date back to the Permian-Triassic boundary (250 million years ago, Ma), some 130 million years earlier than the first silicified fossil diatoms (Gersonde & Harwood 1990, Medlin et al. 1997). At the Eocene-Oligocene boundary (36 Ma), diatoms became a widely diverse group that has steadily risen to ecological prominence in today's ocean (Katz et al. 2004).

Coccolithophores are unicellular planktonic algae that are covered with plates of calcium carbonate ($CaCO_3$), the coccoliths, which contribute to the carbonate pump. The coccoliths constitute approximately half of the current oceanic $CaCO_3$ production (Milliman 1993). There are ~250 species of coccolithophores present in the ocean (Winter & Siesser 1994). This group originated in the late Triassic (~220 Ma, Bown et al. 2004) and led to the significant accumulation of calcite plates in oceanic sediments especially in the late Jurassic (~150 Ma, Morse & Mackenzie 1990). Coccolithophore species diversity declined as a result of several mass extinction events (e.g. Alvarez et al. 1980) while diatom diversity has increased up to the present day.

The occurrence of diatoms and coccolithophores in different ecological niches has been attributed to several physiological adaptations that will be discussed in this paragraph. Numerous diatom species are known to bloom frequently along continental margins and in

upwelling regions where nutrient availability is high (Smetacek 1999). The occurrence of high diatom abundances in nutrient-rich waters has been related to the evolution of a nutrient storage vacuole that allows diatoms to accumulate nutrients in excess of their immediate growth requirements and thereby deprives competing taxa of these essential resources (Raven 1997). In contrast, coccolithophores possess very high affinities for nutrients, such as phosphate, thus providing a physiological adaptation especially in oligotrophic waters. Within the group of coccolithophores, only the two species *Emiliania huxleyi* and *Gephyrocapsa oceanica* form blooms. Both species cannot be regarded as typical coccolithophores in terms of phylogeny and ecology (Sáez et al. 2003) as for instance they bloom in nitrate-rich, but phosphate-poor waters (e.g. Haidar & Thierstein 2001). These blooms predominantly occur in well stratified waters in late spring/early summer, a finding that as been attributed to their unusual tolerance to high light (Nielsen 1995, Nanninga & Tyrrell 1996, Harris et al. 2005). Exposed to high irradiances, diatoms are often photoinhibited while coccolithophores like *E. huxleyi* appear to be resistant to photoinhibition (Nielsen 1997). As a possible explanation for this lack of photoinhibition, it has been proposed that calcification provides a means of energy dissipation under high irradiances (Paasche 2001). Diatom-dominated blooms are mainly found in turbulent, low-stratified waters during early spring season, it appears that this group copes better with changing light intensities and especially low light conditions (Rost & Riebesell 2004, Wagner et al. 2006, Lavaud et al. 2007).

Aside from physiological constraints regarding light, the inorganic carbon (C_i) availability also affects the succession of species (e.g. Hansen 2002). The effect of C_i availability on the phytoplankton community has been largely ignored in phytoplankton ecology. This is mainly due to the fact that dissolved inorganic carbon is always in excess relative to other nutrients. In seawater, C_i is mainly found in the form of HCO_3^- (~2 mmol L^{-1}), but also in low and varying concentrations of dissolved CO_2 (~5-25 μmol L^{-1}). Especially towards the end of bloom periods, phytoplankton cells cause the drawdown of CO_2 due to their high photosynthetic activity. Intense photosynthetic activity can result in pH values as high as ~9 in marine environments (Hinga 2002). Another important aspect that influences phytoplankton structure and growth is the rise in atmospheric CO_2 levels due to human-induced activities such as fossil fuel burning. Present day seawater pCO_2 of 380 μatm are 100 μatm higher than preindustrial values owing to the uptake of 'anthropogenic' CO_2 by the ocean. By the end of this century, it is expected that the seawater pCO_2 increase to 750 μatm and that by then seawater pH will have dropped by 0.4 units relative to

preindustrial values (Fig. 1, Wolf-Gladrow et al. 1999, IPCC 2007). These changes are likely to influence the dominance of diatoms and coccolithophores in the future.

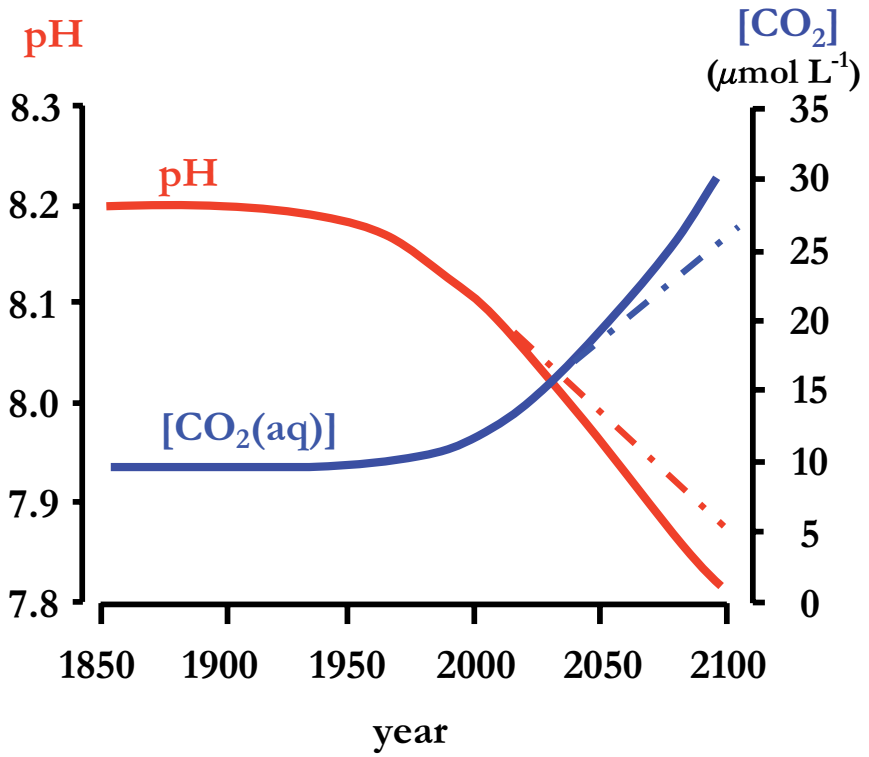

Fig. 1 Seawater pH and the dissolved CO_2 concentration in the surface layer of the ocean assuming a 'business as usual' (IS92a) anthropogenic CO_2 emission scenario (IPCC 2007). Modified after Wolf-Gladrow et al. (1999).

3.2 SEAWATER CARBONATE SYSTEM

When investigating the effects of pH/CO_2 on the carbon acquisition in marine phytoplankton, a good understanding of the seawater carbonate chemistry is required. Gaseous CO_2 (g) dissolves in seawater and following Henry's law the concentration of aqueous CO_2, $[CO_2\ (aq)]$, is proportional to the partial pressure of gaseous CO_2, pCO_2:

$$[CO_2\ (aq)] = \alpha\ pCO_2 \qquad (1)$$

Where α is the temperature- and salinity-dependent CO_2 solubility coefficient, and pCO_2 denotes the partial pressure of CO_2 in the atmosphere. In contrast to the other atmospheric gases like nitrogen and oxygen, the relative amount of dissolved inorganic carbon in seawater is much greater since CO_2 undergoes several chemical reactions. The dissolved CO_2 is hydrated to H_2CO_3 and subsequently dissociates to HCO_3^-, CO_3^{2-} and protons (H^+):

$$CO_2\,(aq) + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \quad (2)$$

Since H_2CO_3 occurs in very small amounts, the sum of H_2CO_3 and CO_2 (aq) is usually denoted as CO_2. The sum of the dissolved carbon species is thus defined as dissolved inorganic carbon (DIC):

$$DIC = [CO_2] + [HCO_3^-] + [CO_3^{2-}] \quad (3)$$

The relative proportions of the three carbon species varies as a function of pH as shown in Fig. 2. At today's seawater pH of 8.2, the [DIC] is ~2200 μmol L^{-1}. HCO_3^- contributes up to ~90% of DIC (2000 μmol L^{-1}) while CO_2 (aq) accounts for less than 1% of DIC (~13 μmol L^{-1}). Thus, HCO_3^- is the dominant carbon species in seawater.

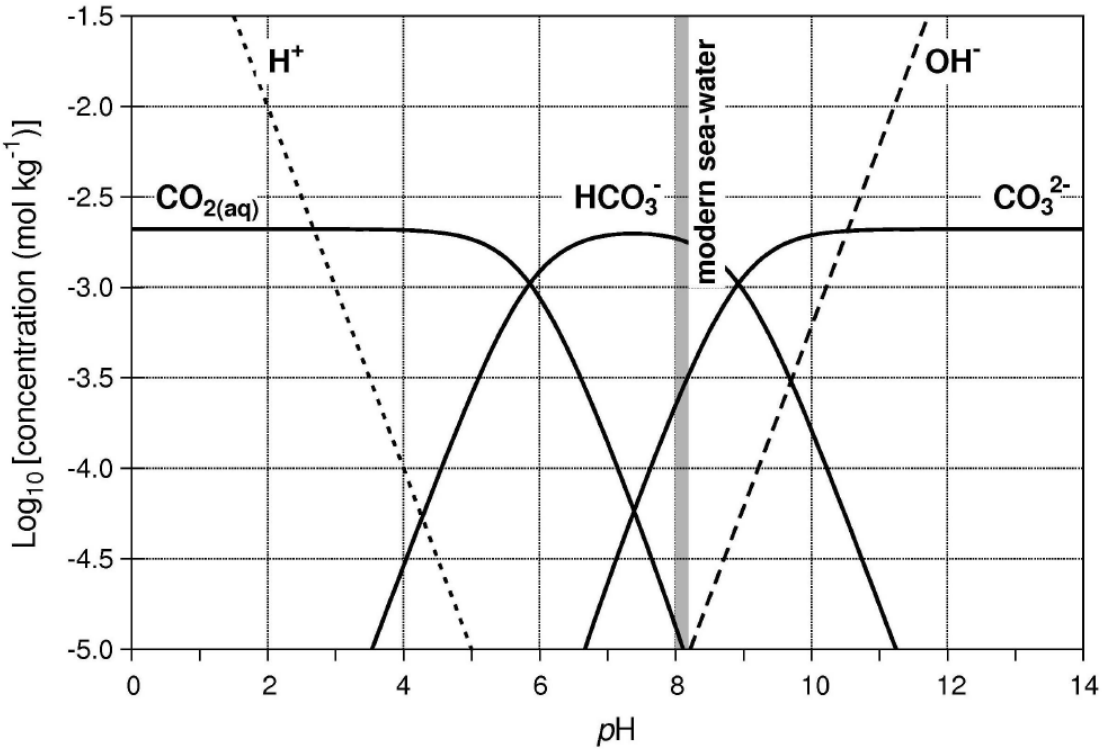

Fig. 2 The [CO_2 (aq)], [HCO_3^-], and [CO_3^{2-}] as a function of pH in seawater. Reproduced from Ridgewell & Zeebe (2005).

Since it is analytically important, the concept of total alkalinity (TA) will be introduced briefly. The amount of TA in seawater can be quantified by means of acidimetric titration (*see* Dickson 1981). Consequently, the higher the alkalinity of seawater the higher is its buffering capacity. TA is defined as:

$$TA = [HCO_3^-] + 2[CO_3^{2-}] + [B(OH)_4^-] + [OH^-] + [HPO_4^{2-}] + 2[PO_4^{3-}] + [H_3SiO_4^-] + [HS^-] + [NH_3] - [H^+]_F - [HSO_4^-] - [HF] - [H_3PO_4] \quad (4)$$

Where $[H^+]_F$ is the free concentration of hydrogen ions. This is only one of the concepts that exist for alkalinity. For more details on the various expressions of alkalinity, I refer to Zeebe & Wolf-Gladrow (2003) and Wolf-Gladrow et al. (2007).

When CO_2/pH manipulation experiments are carried out, one has to bear in mind that it is not possible to manipulate one parameter of the carbonate chemistry without affecting another. Given the knowledge of two variables out of the six variables of the carbonate system (CO_2, HCO_3^-, CO_3^{2-}, pH, DIC, TA) one may calculate the other four. An increase in atmospheric pCO_2 in an open system will lead to an increase of both $[CO_2]$ and DIC, even though TA remains unaffected. If pH is decreased through the addition of acid in a closed system, TA will decrease while $[CO_2]$ will increase and DIC will remain constant.

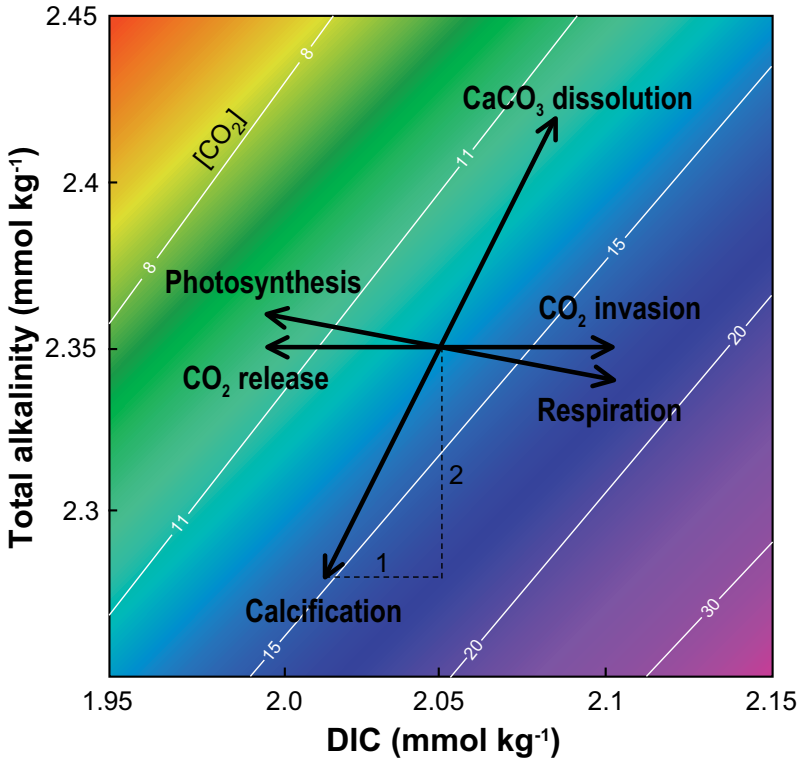

Fig. 3 Effect of processes affecting TA and DIC (arrows; *see* text). Solid and dashed lines indicate levels of constant dissolved CO_2 (in μmol kg^{-1}) and pH, respectively as a function of DIC and TA. Modified after Zeebe & Wolf-Gladrow (2003).

Biological processes can alter the carbonate system as well (Fig. 3). During photosynthesis, CO_2 is taken up by the cells and consequently $[CO_2]$ and DIC decrease, while TA slightly increases because in addition to C_i, nutrients are taken up. The uptake of

one mole nitrate (NO_3^-) goes along with either a simultaneous uptake of H^+ or a release of OH^-, respectively, to ensure electroneutrality. The latter reaction thus leads to an increase in TA. Respiration and remineralisation cause the opposite reactions since CO_2 is released, $[CO_2]$ and DIC will increase while TA decreases. The precipitation of $CaCO_3$ will reduce both DIC and TA, but in a 1:2 ratio. Since CO_2 is produced during the precipitation, $[CO_2]$ will increase. Precipitation and dissolution of $CaCO_3$ depend on the $CaCO_3$ saturation state Ω, which is a function of both the CO_3^{2-} and calcium (Ca^{2+}) ion concentrations:

$$\Omega = \frac{\left[Ca^{2+}\right]_{SW}\left[CO_3^{2-}\right]_{SW}}{K_{sp}^*} \tag{5}$$

Where K_{sp}^* is the solubility product of the respective $CaCO_3$ polymorph (calcite, aragonite or vaterite) at the *in situ* conditions of temperature, salinity, and pressure. Hence, if Ω is larger than 1, the solution is supersaturated with respect to calcite, whereas an Ω of less than 1 corresponds to undersaturation. For more details, please refer to Zeebe & Wolf-Gladrow (2003).

3.3 INORGANIC CARBON ACQUISITION OF MARINE PHYTOPLANKTON

Through the process of photosynthesis marine phytoplankton use the light energy trapped by chlorophyll to oxidise water (H_2O) hereby yielding molecular oxygen (O_2) and synthesising ATP and NADPH in the so-called light reaction (eq. 6). This process is one of the two reactions in photosynthesis. In the other reaction, called the dark reaction, CO_2 is reduced to carbohydrates by the use of ATP and the reducing agent NADPH:

$$2H_2O + CO_2 + \text{Light} \xrightarrow{\text{Chl}} (CH_2O) + H_2O + O_2 \tag{6}$$

The reduction of CO_2 is catalysed by Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). RubisCO requires CO_2 as substrate, but in contrast to the high HCO_3^- concentrations in the contemporary ocean (90%) of DIC, CO_2 is rare (1% of DIC). Moreover, RubisCO has only a slow maximum turnover rate as well as a poor affinity for its substrate (K_M of 20-70 μmol L^{-1} for marine phytoplankton, Badger et al. 1998). This is partially due to the fact that RubisCO also reacts with O_2. This reaction initiates the photorespiration process, which finally lowers the photosynthetic rates. Therefore, at typical CO_2 concentrations in seawater that range between 8 and 20 μmol CO_2 L^{-1} RubisCO

operates far below its optimum (Fig. 4). Considering that most microalgal species thus far investigated have apparent half-saturation concentration ($K_{1/2}$) values for CO_2 of <1-10 μmol L^{-1} (Fig. 4, Raven & Johnston 1991), which are much lower than those of RubisCO (Badger et al. 1998), there is strong evidence that most marine microalgae actively take up inorganic carbon (C_i). They operate so-called carbon concentrating mechanisms (CCMs) that overcome the potential carbon limitation through the enrichment of CO_2 at the catalytic site of RubisCO and thus enhance their photosynthetic productivity. In principle, CCMs of mircoalgae involve active uptake of CO_2 and/or HCO_3^- into the algal cell and/or the chloroplast. The enzyme carbonic anhydrase (CA), which accelerates the otherwise slow interconversion between HCO_3^- and CO_2, is located both inside the cell and at the cell surface. With these basic components microalgae have evolved a large diversity of CCMs (Badger et al. 1998, Tortell 2000, Giordano et al. 2005). This thesis mainly addresses the C_i acquisition of marine diatoms as well as a specific aspect of C_i acquisition of coccolithophores. I will describe the different components of CCMs in the following (*see* Table 1, Fig. 5-6).

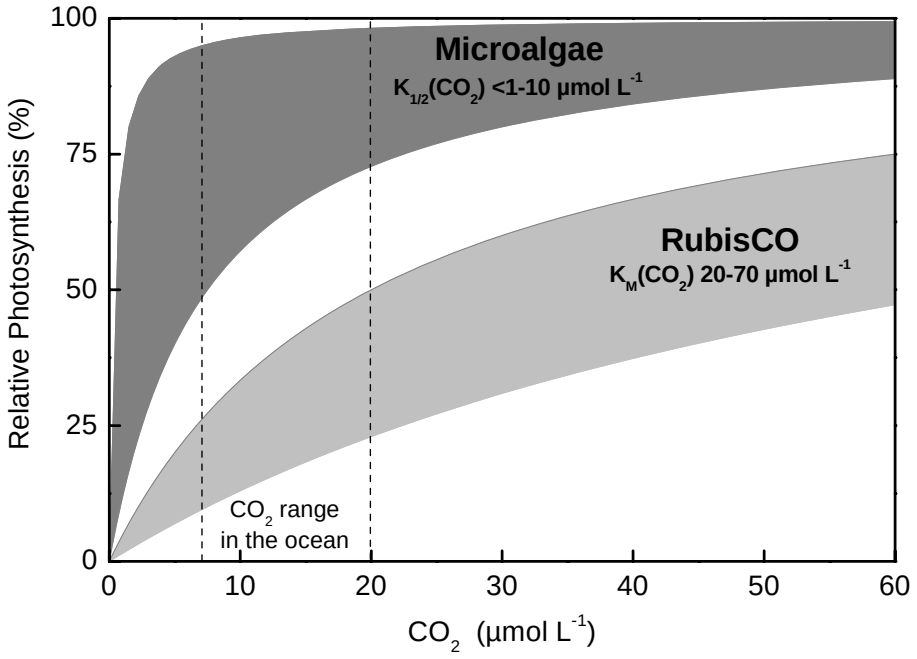

Fig. 4 Comparison of CO_2 fixation between marine phytoplankton cells and isolated RubisCO. Apparent half-saturation constants ($K_{1/2}$) for photosynthetic CO_2 fixation with the half-saturation constant (K_M) of RubisCO, the presence and the efficiency of a CCM can be assessed.

The C_i accumulation in the cells relative to ambient concentrations has been demonstrated by means of the silicon-oil centrifugation technique for diatoms (Burns & Beardall 1987, Colman & Rotatore 1995, Mitchell & Beardall 1996, Tortell et al. 1997) and coccolithophores (Nimer & Merrett 1992, Sekino & Shiraiwa 1994). By comparing the apparent half-saturation constants ($K_{1/2}$) for photosynthetic CO_2 fixation with the half-saturation constant (K_M) of RubisCO (*see* Fig. 4), the presence and the efficiency of a CCM has been assessed in diatoms (Burns & Beardall 1987, Colman & Rotatore 1995, Mitchell & Beardall 1996, Tortell et al. 1997, Burkhardt et al. 2001, Rost et al. 2003) and coccolithophores (Sekino & Shiraiwa 1994, Israel & González 1996, Rost et al. 2003).

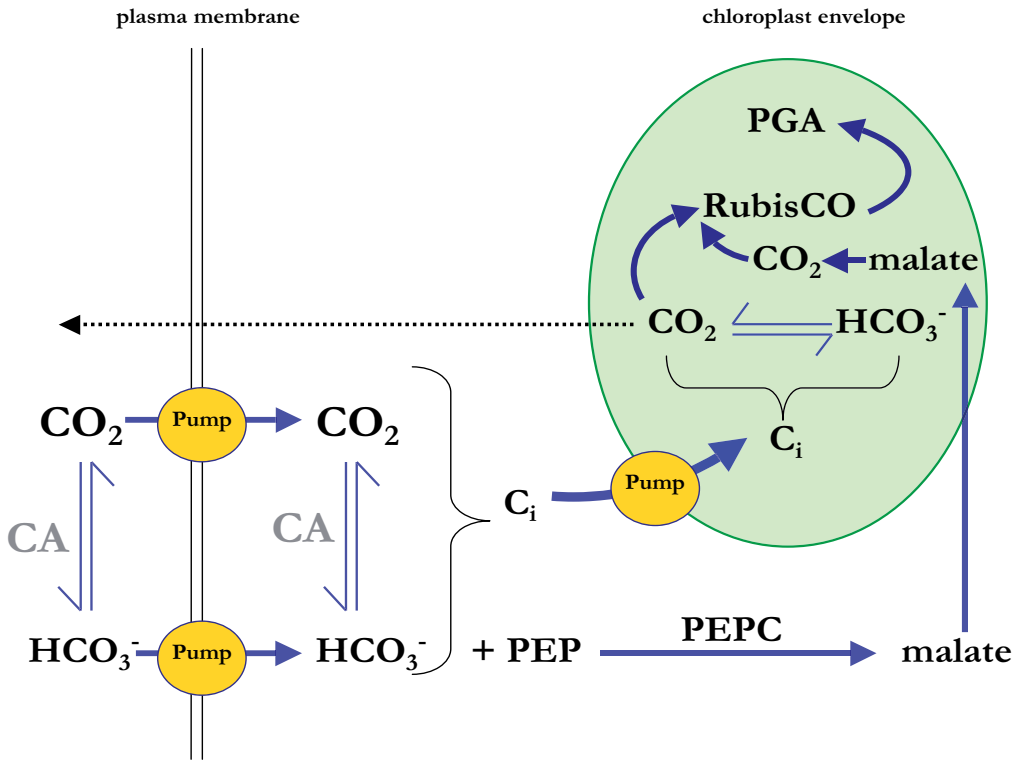

Fig. 5 Schematic presentation of components of a possible CCM in marine diatoms (Table 1 and *see* text).

Under low CO_2 conditions, the ability to actively take up HCO_3^- is of crucial importance and may provide species with a competitive advantage over those that rely on CO_2 as the only C_i source (Nimer et al. 1997, Korb et al. 1997, Hansen 2002, Tortell & Morel 2002). Active influxes of CO_2 and HCO_3^- into the cell have been demonstrated not only for marine diatoms (Burns & Beardall 1987, Colman & Rotatore 1995, Rotatore et al. 1995, Mitchell & Beardall 1996, Korb et al. 1997, Elzenga et al. 2000, Burkhardt et al. 2001, Rost et al. 2003, Rost et al. 2007), but also for coccolithophores (Elzenga et al. 2000, Rost et

al. 2003). Several methodological approaches exist to determine whether CO_2, HCO_3^-, or both carbon species are taken up (Table 1, Fig. 5-6).

The use of HCO_3^- as a carbon source for the three diatoms *Phaeodactlyum tricornutum*, *Cyclotella* sp. and *Nitzschia frigida* was indicated by the observation that the photosynthetic rate of O_2 evolution exceeded the uncatalysed rate of conversion of HCO_3^- and CO_2 in the medium (Colman & Rotatore 1995, Burns & Beardall 1987, Mitchell & Beardall 1996). By application of the ^{14}C disequilibrium technique, the use of both carbon sources in several diatom species and the coccolithophore *Emiliania huxleyi* was shown (Korb et al. 1997, Elzenga et al. 2000, Tortell & Morel 2002, Rost et al. 2007). This approach established by Espie and Colman (1986) uses the transient isotopic disequilibrium upon a ^{14}C spike to determine whether CO_2 or HCO_3^- is the preferred carbon species for photosynthesis.

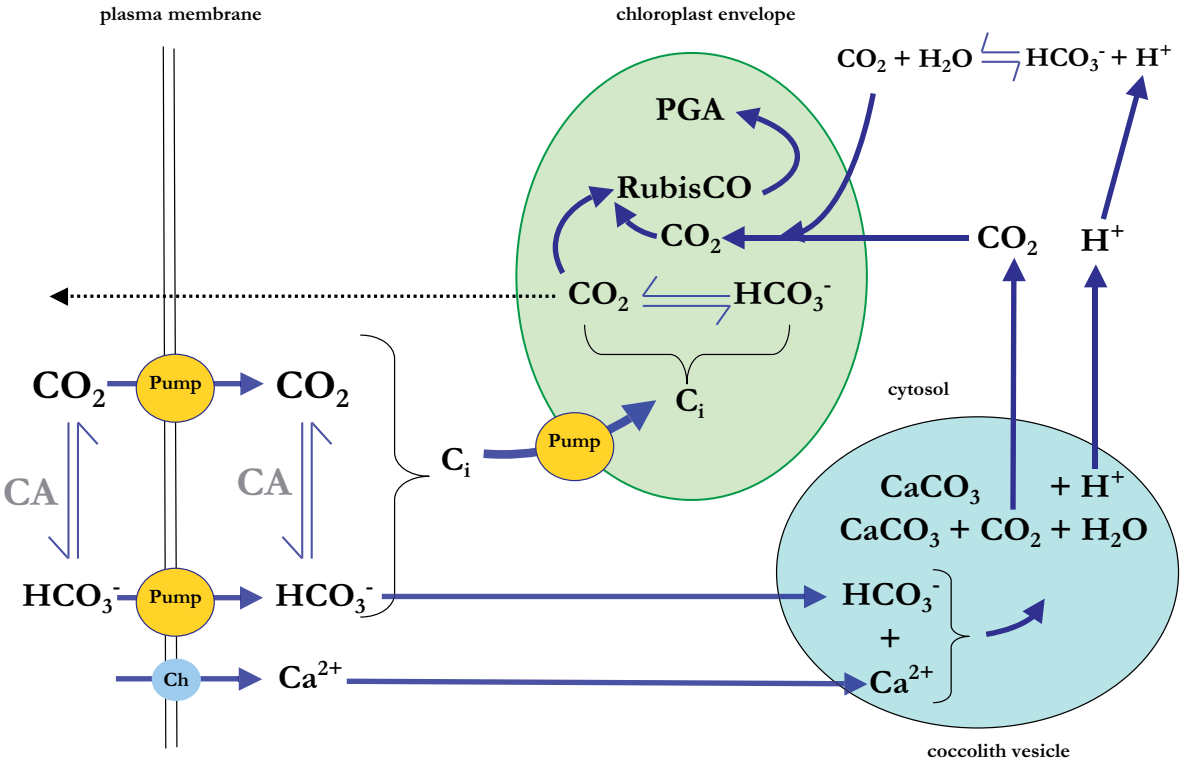

Fig. 6 Schematic presentation of components of a possible CCM in coccolithophores (Table 1 and text). Calcium (Ca^{2+}) diffuses into the cell, most likely through Ca^{2+}-selective channels (Ch) in the plasma membrane (Brownlee & Sanders 1992). The specific transport mechanism of Ca^{2+} and HCO_3^- into the coccolith vesicle will not be discussed here as this is not the subject of this thesis. In the coccolith vesicle, $CaCO_3$ is produced either by the formation of CO_2 and H_2O following eq. 7 or H^+ following eq. 8 (*see* text).

Using membrane inlet mass spectrometry (MIMS), Rotatore et al. (1995) monitored the initial CO_2 depletion and the photosynthetic O_2 evolution by the two diatoms *P. tricornutum* and *Cyclotella* sp. upon illumination. Under non-steady-state photosynthesis, the

depletion of C_i was found to exceed the rate of CO_2 uptake, it was concluded that both diatoms use HCO_3^- in addition to CO_2. The more refined MIMS approach established by Badger et al. (1994) uses the chemical disequilibrium between CO_2 and HCO_3^- during steady-state photosynthesis, i.e. the rate of CO_2 and HCO_3^- uptake and the O_2 evolution rate are constant. Thus it allows CO_2 and HCO_3^- fluxes across the plasmalemma to be quantified. C_i flux estimates are based on simultaneous measurements of O_2 and CO_2 during consecutive light and dark intervals. The addition of increasing amounts of C_i to an initially CO_2-free assay buffer provides kinetics for photosynthesis and for the uptake of HCO_3^- and CO_2. This approach has been applied for marine diatoms *Thalassiosira weissflogii* (Burkhardt et al. 2001), *P. tricornutum* (Burkhardt et al. 2001), *Skeletonema costatum* (Rost et al. 2003), *Thalassionema nitzschiodes* (Rost et al. 2007) and the coccolithophore *Emiliania huxleyi* (Rost et al. 2003). Rost et al. (2007) demonstrated that the ^{14}C disequilibrium technique and the MIMS approach following Badger et al. (1994) provide nearly identical results on the contribution of HCO_3^- and CO_2 relative to net carbon fixation. The active uptake of CO_2 and HCO_3^- at the chloroplast envelope was shown for green algae (Amoroso et al. 1998, Hunnik et al. 2002). Similar findings might be expected for marine diatoms and coccolithophores, but it still needs to be experimentally verified (Table 1, Fig. 5-6). This could be done by means of MIMS technique for isolated chloroplasts of diatoms and coccolithophores (*see* Amoroso et al. 1998, Hunnik et al. 2002).

The enzyme carbonic anhydrase (CA) accelerates the otherwise slow interconversion rate between HCO_3^- and CO_2 either at the cell surface or inside the cell (Table 1, Fig. 5-6). It is a common notion that eCA increases the CO_2 concentration in the boundary layer of the cell by converting HCO_3^- to CO_2 and herewith favouring CO_2 uptake (Badger & Price 1994, Sültemeyer 1998, Elzenga et al. 2000, Badger 2003). However, model calculations indicate that eCA activities may be insufficient to significantly enhance C_i uptake in marine microalgae (Wolf-Gladrow & Riebesell 1997). The physiological role of eCA in marine diatoms will be discussed in more detail in publication II. Intracellular carbonic anhydrase (iCA) can be located in the cytosol, the mitochondrium and the chloroplast. The function of the multiple iCA forms is still poorly understood (Sültemeyer 1998), but iCA is possibly involved in different processes (Table 1, Fig. 5-6, Badger and Price 1994, Sültemeyer 1998, Badger 2003).

The occurrence of eCA and iCA activities in coccolithophores and diatoms has been shown by several authors applying different methodological approaches (Table 1). The potentiometric method by Wilbur & Anderson (1948) and modifications thereof (Miyachi et al. 1983, Williams & Colman 1993) have been applied to determine eCA and/or iCA

activities in diatoms (Burns & Beardall 1987, Mitchell & Beardall 1996, Nimer et al. 1997) and coccolithophores (Nimer et al. 1997). This approach is based on the time-dependent pH decrease upon a CO_2 saturated spike of distilled water in the presence and the absence of cells and/or a cell extract, respectively. The CA activity of cell extracts can be taken as a measure of total CA activity (eCA and iCA), and the internal activity can be calculated as the difference between total activity and that of intact cells (eCA). The mass spectrometric approach by Silverman (1982) has been applied by Burkhardt et al. (2001) and Rost et al. (2003, 2007) for diatoms and coccolithophores. This method enables the determination of CA activities in very low cell densities by measuring the loss of ^{18}O from doubly labelled $^{13}C^{18}O_2$ to water caused by the interconversion of CO_2 and HCO_3^- (Silverman 1982, Tu et al. 1986, Sültemeyer et al. 1990, Palmqvist et al. 1994). This approach has the advantage that not only eCA but also iCA activities can be measured in living cells. For more details on the assumptions underlying the applied technique I refer to Palmqvist et al. (1994) and Rost et al. (2003). By use of the modified approach of the ^{14}C disequilibrium technique (Elzenga et al. 2000), eCA activities are indirectly derived by comparing the ^{14}C uptake kinetics in the absence and presence of dextran-bound sulfonamide (DBS), an inhibitor for eCA. Note that this approach provides reasonable estimates of eCA activities, but only when cells are not predominantly HCO_3^- users and possess low amounts of eCA (Rost et al. 2007). By means of this approach, evidence for eCA activity was found for the lowly-calcified *E. huxleyi* (Elzenga et al. 2000) and *T. nitzschioides* (Rost et al. 2007).

For diatoms, C_4-like photosynthesis has been considered as an additional strategy to ensure efficient photosynthesis under low CO_2 (Reinfelder et al. 2000, 2004, Table 1, Fig. 5). While most microalgae and terrestrial plants use the C_3 pathway and directly incorporate CO_2 by RubisCO into a 3-phosphoglyceric acid (PGA, a C_3 compound), some higher land plants like maize have evolved the C_4 pathway. In C_4 plants, CO_2 is first fixed by the enzyme phosphoenolpyruvate-carboxylase (PEPC) into a C_4 compound (instead of C_3) that is decarboxlated again in close vicinity of RubisCO. In principle, CO_2 enters the mesophyll cells of the leaf by diffusion and is subsequently catalysed by CA to HCO_3^- which is then carboxylated by PEPC to the C_4 compound oxalacetate (OAA). Owing to its high affinity to its substrate HCO_3^- and the lack of the oxygenase activity, PEPC is far more efficient in carbon fixation than RubisCO. OAA is then reduced to malate and transported into the chloroplast of the bundle sheath cells where malate is decarboxylated to pyruvate and CO_2. The latter is then finally fixed by RubisCO. It was a common notion that the C_4 metabolism was absent in marine microalgae until Reinfelder et al. (2000) found evidence for C_4-like

Table 1 Summary of possible components of a CCM and their location in marine diatoms and coccolithophores. The methodological approaches to investigate one or more components of the CCM and studies that have employed these approaches on diatoms and coccolithophores are given as well. Modified from Giordano et al. 2005.

Component of CCM	Location in cell	Occurrence in diatoms?	Occurrence in coccolithophores?	Methodological approach	References
Active influx of CO_2	Plasmalemma and/or chloroplast envelope	Yes	Yes	- Measurements of O_2 evolution rate in response to varying DIC concentrations - Silicon-oil centrifugation technique - ^{14}C disequilibrium technique - MIMS approach	Burns & Beardall 1987, Colman & Rotatore 1995, Rotatore et al. 1995, Mitchell & Beardall 1996, Korb et al. 1997, Elzenga et al. 2000, Burkhardt et al. 2001, Tortell & Morel 2002, Rost et al. 2003, Rost et al. 2007
Active influx of HCO_3^-	Plasmalemma and/or chloroplast envelope	Yes	Yes		
eCA	Periplasmic space	Yes	Yes	- Potentiometric approach and modifications thereof - Mass spectrometric approach - Modified ^{14}C disequilibrium technique	Burns & Beardall 1987, Mitchell & Beardall 1996, Nimer et al. 1997, Elzenga et al. 2000, Burkhardt et al. 2001, Rost et al. 2003, Rost et al. 2007
iCA	Cytosol, mitochondrium, chloroplast	Yes	Yes	- Potentiometric approach and modifications thereof - Mass spectrometric approach	Burns & Beardall 1987, Mitchell & Beardall 1996, Burkhardt et al. 2001, Rost et al. 2003
C_4-like photosynthetic pathway	C_3 compound carboxylated in cytosol, decarboxylation of C_4 compound in chloroplast in close proximity to RubisCO	Confirmed for *T. weissflogii*, not valid for *T. pseudonana*	No	- ^{14}C labelling experiments - Pulse-chase experiments - Inhibitor studies - RT-PCR	Reinfelder et al. 2000, Reinfelder et al. 2004, Morel et al. 2002, Granum et al. 2005, Roberts et al. 2007, McGinn & Morel 2008
Calcification process releases CO_2 that supports carbon fixation	Calcification in coccolith vesicle, H^+ or CO_2 transported in chloroplast	No	controversially discussed	- ^{14}C incorporation during photosynthesis and calcification - Measurements of O_2 evolution rate in response to varying DIC concentrations	Sikes et al. 1980, Paasche 1964, Herfort et al. 2002, 2004

photosynthesis in the marine diatom *T. weissflogii* (Table 1, Fig. 5). They suggested that PEPC is the primary carboxylase in the cytoplasm that forms C_4 compounds from PEP and HCO_3^-.

The C_4 compound malate (possibly also aspartate) is then transported into the chloroplast and decarboxylated by phosphoenolpyruvate-carboxykinase (PEPCK) in close proximity of RubisCO to support carbon fixation (Fig. 5).

Reinfelder et al. (2000) performed short-term ^{14}C-labelling experiments and found that the first acid-stable product of ^{14}C incorporation was the C_4 compound malate rather than PGA indicating the operation of a C_4-like pathway. This observation has been confirmed for *T. weissflogii* by Roberts et al. (2007). Pulse-chase experiments with *T. weissflogii* demonstrated that during the first 60s the ^{14}C activity in malate declines while there was an increase in the ^{14}C content of PGA (Reinfelder et al. 2000, Morel et al. 2002). Further support for C_4-like photosynthesis in *T. weissflogii* comes from the observation that the addition of an inhibitor for PEPC resulted in a significant decline of the photosynthetic O_2 evolution (Reinfelder et al. 2004). RT-PCR as well as ^{14}C short-term labelling experiments did not reveal C_4-like metabolism in the marine diatom *T. pseudonana* (Granum et al. 2005, Roberts et al. 2007). In contrast to this, McGinn and Morel (2008) found evidence for C_4 photosynthesis in the same strain of *T. pseudonana* based on analysis of gene transcripts of PEPC and PEPCK and inhibitor studies of these enzymes. The occurrence of a C_4-like photosynthetic pathway in *T. pseudonana* as well as in other selected diatom species will be discussed in Publication III.

For coccolithophores it has been speculated that the process of calcification is involved in photosynthetic carbon acquisition (Sikes et al. 1980, Table 1, Fig. 6). Provided that HCO_3^- is the carbon source for calcification (*see* Paasche 2001), calcification could promote photosynthesis by supplying CO_2 or protons (H^+) according to the following reactions:

$$Ca^{2+} + 2\ HCO_3^- \rightarrow CaCO_3 + CO_2 + H_2O \quad (7)$$

$$Ca^{2+} + HCO_3^- \rightarrow CaCO_3 + H^+ \quad (8)$$

While CO_2 could then be used directly in photosynthesis, H^+ released from the coccolith vesicle may react with HCO_3^- in the cytosol to produce CO_2 (Fig. 6). Such a mechanism would allow coccolithophores to access the abundant HCO_3^- pool in seawater and provide them with an intracellular source of CO_2. Hence, coccolithophores would have an advantage, especially under low CO_2 concentrations. According to Anning et al. (1996), such functional coupling could represent a cost-efficient alternative to a classical CCM. The

involvement of calcification in photosynthetic carbon acquisition is still under debate (e.g. Paasche 2001, Rost & Riebesell 2004) and will be discussed in publication I.

In view of the ongoing acidification of the oceans (Wolf-Gladrow et al. 1999, IPCC 2007) as well as elevated pH during blooms (Hansen 2002), differences in CCM efficiency and regulation are likely to affect the ecological fitness of diatoms and coccolithophores and might influence the dominance of species (Tortell et al. 2002, Rost et al. 2003). Species that are able to use the large HCO_3^- pool may have a competitive advantage over those that rely on CO_2 and thus may be less sensitive to variations in pH (Nimer et al. 1997, Korb et al. 1997, Hansen 2002, Tortell & Morel 2002). During bloom situations in particular, diatoms and coccolithophores have to cope with decreasing CO_2 concentration owing to high photosynthetic activity. To this end, the ability to strongly regulate the C_i acquisition may provide a physiological adaptation to maintain high growth rates during bloom situations (Rost et al. 2003). Under these conditions, diatoms operating a C_4-like photosynthetic pathway might have a competitive advantage (Reinfelder et al. 2000, 2004, Morel et al. 2002, Roberts et al. 2007, McGinn & Morel 2008) while for coccolithophores calcification would provide an additional source of CO_2 thus counteracting decreasing CO_2 supply (Sikes et al. 1980, Anning et al. 1996). Rising CO_2 levels due to anthropogenic input have already affected seawater carbonate chemistry. Present day surface ocean pH is ~0.1 units lower than preindustrial values. It is well known that CCMs in diatoms are sensitive to changing CO_2 concentrations (Burkhardt et al. 2001, Rost et al. 2003). The response to altered carbonate chemistry is likely to vary among diatom species. A better understanding of species-specific differences in efficiency and regulation of carbon acquisition may allow the prediction of changes in the community structure with regard to the projected increase in CO_2.

3.4 OUTLINE OF THE THESIS

This thesis investigates the C_i acquisition in marine phytoplankton with emphasis on selected diatom species and the role of calcification in the photosynthetic carbon acquisition of *E. huxleyi*. The efficiency and regulation of the CCM in diatoms in response to varying carbonate chemistry were studied in laboratory as well as in field experiments.

Publication I addresses the questions whether calcification is involved in the photosynthetic carbon acquisition in the coccolithophore *E. huxleyi* and whether calcification is a means of energy dissipation under high irradiances.

Publication II investigates the effect of the pH-induced changes in seawater carbonate chemistry on carbon acquisition of toxic and non-toxic diatoms. The ecological implications of certain modes of carbon acquisition in diatoms are assessed in relation to their pH limits of growth and a novel role for eCA is proposed.

Publication III considers the carbon acquisition in relation to the CO_2 supply in four marine diatom species. The questions whether modes of carbon acquisition differ in bloom-forming diatoms and whether the investigated species rely on the C_4-like photosynthetic pathway are addressed.

Publication IV examines the effect of CO_2 concentrations on the C_i uptake, growth and species composition of phytoplankton assemblages in the Ross Sea, Antarctica. The effects of elevated CO_2 on the carbon acquisition of phytoplankton populations during ship-board incubation experiments are discussed.

In a concluding discussion, main results of this thesis are summarised and evaluated with respect to the ecological relevance of certain components of CCMs among marine diatoms and specific aspects in marine coccolithophores. Possible impacts of CO_2/pH-related changes in seawater carbonate chemistry on the ecology of marine diatoms will be assessed. Finally, perspectives are given for future research.

4 PUBLICATIONS

4 PUBLICATIONS

4.1 LIST OF PUBLICATIONS

This doctoral thesis is based on the following publications:

I. Scarlett Trimborn, Gerald Langer and Björn Rost. 2007. Effect of varying calcium concentrations and light intensities on calcification and photosynthesis in *Emiliania huxleyi*. *Limnology and Oceanography* 52(5): 2285-2293.

II. Scarlett Trimborn, Nina Lundholm, Silke Thoms, Bernd Krock, Klaus-Uwe Richter, Per J. Hansen and Björn Rost. 2008. Inorganic carbon acquisition in potentially toxic and non-toxic diatoms: the effect of pH-induced changes in carbonate chemistry. *Physiologia Plantarum* doi: 10.1111/j.1399-3054.2007.01038.x.

III. Scarlett Trimborn, Dieter Wolf-Gladrow, Klaus-Uwe Richter and Björn Rost. The effect of pCO_2 on the carbon acquisition and intracellular assimilation in four marine diatom species. Submitted to *Journal of Phycology*.

IV. Philippe D. Tortell, Christopher D. Payne, Yingyu Li, Scarlett Trimborn, Björn Rost, Walker O. Smith, Christina Riesselman, Robert B. Dunbar, Pete Sedwick and Giacomo R. DiTullio. The CO_2 sensitivity of Southern Ocean phytoplankton. *Geophysical Research Letters*, *In press*.

4.2 DECLARATION ON THE CONTRIBUTION OF EACH PUBLICATION

Publikation I

Die Laborexperimente wurden von mir geplant und durchgeführt. Ich habe die Daten ausgewertet und das Manuskript in Zusammenarbeit mit den Koautoren verfasst.

Publikation II

Die Laborexperimente wurden in Zusammenarbeit mit Nina Lundholm geplant und durchgeführt. Ich habe die Daten ausgewertet und das Manuskript in Zusammenarbeit mit den Koautoren verfasst.

Publikation III

Die Laborexperimente wurden von mir geplant und durchgeführt. Ich habe die Daten ausgewertet und das Manuskript in Zusammenarbeit mit den Koautoren verfasst.

Publikation IV

Die Feldexperimente wurden von Philippe Tortell geplant und in Zusammenarbeit mit den Koautoren durchgeführt und ausgewertet. Das Manuskript wurde in Zusammenarbeit mit den Koautoren verfasst.

PUBLICATION I

Limnol. Oceanogr., 52(5), 2007, 2285–2293

Effect of varying calcium concentrations and light intensities on calcification and photosynthesis in *Emiliania huxleyi*

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Abstract

Various protective and metabolic functions for coccolithophore calcification have been proposed such as providing a means to supply CO_2 for photosynthesis. It has also been speculated that calcification helps to dissipate excess energy under high irradiance, thereby circumventing photoinhibition. To address these questions, cells of a calcifying strain of *Emiliania huxleyi* were grown at three irradiances (30, 300, and 800 μmol photons m^{-2} s^{-1}) in combination with four calcium (Ca) concentrations (0.1, 1, 2.5, and 10 mmol L^{-1}) leading to different degrees of calcification in the same strain. Growth rates (μ), particulate organic carbon (POC), and inorganic carbon (PIC) production as well as carbon isotope fractionation (ε_p) were determined. Photosynthetic O_2 evolution and CO_2 and HCO_3^- uptake rates were measured by membrane inlet mass spectrometry (MIMS). The application of this multimethod approach provides new information on the role of calcification in *E. huxleyi*. Noncalcifying cells showed POC production rates as high as calcifying ones. No differences in ε_p were observed under different Ca concentrations. MIMS measurements indicate that noncalcifying cells can photosynthesize as efficiently as, or even more efficiently than, calcifying ones and that both use HCO_3^- as the main carbon source. The ratio of photosynthetic HCO_3^- uptake relative to net fixation did not differ among cells acclimated to 10 mmol L^{-1} or to 0.1 mmol L^{-1} Ca. These results indicate that (1) calcification is not involved in photosynthetic carbon acquisition, and (2) calcification does not provide a means of energy dissipation under high irradiances.

Marine phytoplankton are a key player in driving the biological carbon pumps. Whereas diatoms only affect the organic carbon pump through the process of photosynthesis, calcifiers like *Emiliania huxleyi* additionally influence the carbonate pump by producing calcium carbonate. *E. huxleyi* is the most abundant coccolithophore in the oceans, distributed worldwide apart from the polar regions (Winter et al. 1994). It is the best-studied coccolithophore species, although it cannot be regarded as a typical coccolithophore in terms of phylogeny (Sáez et al. 2003). An uncommon trait of *E. huxleyi* is its ability to form extensive blooms with densities up to 10^7 cells L^{-1} (Holligan et al. 1993). Such blooms can be observed mainly in early summertime (Balch et al. 1991) when the water column becomes stratified and the mixed water layer depth is 30 m at most (e.g., Nanninga and Tyrrell 1996). This is in contrast to diatom blooms, which mainly form in springtime when the upper ocean is deeply mixed.

Light saturation for growth is comparably high in *E. huxleyi* compared with diatoms (Richardson et al. 1983). Exposed to high irradiances, diatoms are often photoinhibited, whereas *E. huxleyi* appears to be resistant to photoinhibition (Nielsen 1997). Only a few studies have investigated this unusual tolerance for high irradiances in *E. huxleyi* (e.g., Nanninga and Tyrell 1996; Harris et al. 2005). According to their findings, coccoliths do not act as "protective light screens." An alternative, yet not rigorously tested, explanation for the lack of photoinhibition has been proposed, i.e., calcification may serve as a means to dissipate excess energy when exposed to high light levels (Paasche 2001). In addition to this, other metabolic functions of calcification have been hypothesized for *E. huxleyi* (Young 1994; Paasche 2001).

Sikes et al. (1980) proposed that the process of calcification is involved in photosynthetic carbon acquisition. Provided that HCO_3^- is the carbon source, calcification could promote photosynthesis by supplying CO_2 or protons H^+ according to the following reactions:

$$Ca^{2+} + 2HCO_3^- \rightarrow CaCO_3 + CO_2 + H_2O \quad (1)$$

$$Ca^{2+} + HCO_3^- \rightarrow CaCO_3 + H^+ \quad (2)$$

CO_2 could then be used directly in photosynthesis or protons could be used in the conversion of HCO_3^- to CO_2. Supported by the observation that the carbon isotope composition of coccoliths is similar to the carbon isotope composition of HCO_3^- (Sikes and Wilbur 1982; Rost et al. 2002), it is generally accepted that HCO_3^- is the carbon source for calcification (*see* Paasche 2001). Such functional coupling would therefore allow the cell to access HCO_3^-, which represents the largest pool of inorganic carbon in seawater, providing an advantage especially under low CO_2 concentrations.

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According to Anning et al. (1996) this mechanism could represent a cost-efficient alternative to a classical carbon concentrating mechanism (CCM) found in microalgae (*see* Badger et al. 1998). CCMs involve active uptake of CO_2 or HCO_3^- (or both) to enhance the CO_2 concentration within the cell, thereby compensating for the low CO_2 affinities of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). On the basis of low affinities for inorganic carbon in *E. huxleyi*, which were only slightly higher than those observed for RubisCO, it has been assumed that this species takes up CO_2 for photosynthesis only via diffusion (Raven and Johnston 1991). Sekino and Shiraiwa (1994) demonstrated, however, that *E. huxleyi* is able to accumulate dissolved inorganic carbon (DIC) to more than 10-fold the ambient seawater concentrations. This is in accordance with Rost et al. (2003), who found that *E. huxleyi* operates a rather inefficient yet actively regulated carbon acquisition.

The involvement of calcification in photosynthetic carbon acquisition has been discussed controversially (e.g., Young 1994; Paasche 2001; Rost and Riebesell 2004); however, there is increasing evidence that the two processes are not coupled (Paasche 1964; Balch et al. 1996; Herfort et al. 2002, 2004). Paasche (1964) demonstrated that photosynthesis is not affected when the cells are transferred to Ca-free medium, a finding that was confirmed more recently by Herfort et al. (2002). These studies stand in contrast to those of Nimer et al. (1996) who found reduced photosynthetic rates by removal of external calcium.

In the present study two issues are addressed: (1) does calcification play a role in the high light tolerance of *E. huxleyi*? and (2) does photosynthesis benefit from calcification? To address these two questions, cells of a calcifying strain of *E. huxleyi* were grown under three light intensities (30, 300, and 800 μmol photons m^{-2} s^{-1}) in combination with four calcium (Ca) concentrations (0.1, 1, 2.5, and 10 mmol L^{-1}). This experimental setup allowed new information to be obtained on the role of calcification in a calcifying strain, in which both the degree of calcification (via Ca concentration) and photosynthesis (via light intensity) were varied. We assessed the relation between photosynthesis and calcification by determining growth rates, particulate organic carbon (POC) and calcite (PIC) production, as well as ^{13}C fractionation. Scanning electron microscopy (SEM) allowed us to examine the relation between the calcification rates and the morphology of the cells. The carbon sources taken up for photosynthesis were determined by membrane-inlet mass spectrometric (MIMS) measurements.

Material and methods

Culture conditions and sampling—A coccolith-bearing strain of *E. huxleyi* (B92/11) was grown in dilute batch cultures in sterile-filtered (0.2 μm) artificial seawater with four different Ca concentrations and three different incident photon flux densities (PFDs). The Ca concentrations, ranging from 0.1 to 10 mmol L^{-1}, were adjusted by addition of $CaCl_2$. The growth medium was enriched with trace metals and vitamins according to f/2 medium (Guillard and Ryther 1962) and nitrate and phosphate concentrations of 100 and 6.25 μmol L^{-1}, respectively. The detailed composition of the seawater is given in Langer et al. (2006). The pH was adjusted to a value of 8.24 by the addition of 0.5 NaOH (mol L^{-1}). Experiments were carried out under a light : dark (LD) cycle of 16 : 8 h at a constant temperature of 15°C. Three photon flux densities (30, 300, and 800 μmol photons m^{-2} s^{-1}) were applied in an incubator (Rubarth Apparate) with daylight fluorescence lamps providing a spectrum similar to that of sunlight. Each treatment was incubated in triplicate in HCl-rinsed polycarbonate flasks. Gentle rotation of the culture flasks three times a day ensured that the cells were kept in suspension.

Cells were acclimated to experimental conditions (including respective Ca concentrations) for at least 10 d, ensuring exponential growth before inoculation. To prevent consumption of more than 7% DIC during the experiment, cells were harvested at low cell densities of around 60,000 cells mL^{-1}. The experiments lasted between 5 and 10 d depending on the experimental conditions. Alkalinity samples were taken from the filtrate (Whatman GFF filter; ~0.6 μm), stored in 300-mL borosilicate flasks at 4°C, and measured in triplicate by potentiometric titration with an average precision of ±8 μeq kg^{-1} (Brewer et al. 1986). Total alkalinity was calculated from linear Gran plots (Gran 1952). DIC samples were sterile-filtered (0.2 μm) and stored in 13-mL borosilicate flasks free of air bubbles at 4°C until they were measured with a Shimadzu TOC 5050A with an average precision of ±17 μmol kg^{-1}. The carbonate system was calculated from alkalinity, DIC, phosphate, temperature, and salinity using the program CO2Sys (Lewis and Wallace 1998). Equilibrium constants of Mehrbach et al. (1973) refitted by Dickson and Millero (1987) were chosen. Detailed information on the parameters of the carbonate system is given in Table 1. The carbonate chemistry did not vary significantly between different flasks. In our experiments the calcite saturation product Ω was changed via the Ca concentration and not through the CO_3^{2-} concentration; the latter one as well as the pH were kept constant (see Table 1). Hence, the seawater carbonate chemistry was not changed in our experimental setup and is similar to values obtained in natural seawater.

Samples for total particulate carbon (TPC) and POC were filtered onto precombusted (500°C; 12 h) GFF filters and stored in precombusted petri dishes (500°C; 12 h) at −20°C. Before analysis, POC filters were treated with 200 μL of HCl (0.1 mol L^{-1}) to remove all the inorganic carbon. TPC, POC, and related $\delta^{13}C$ values were subsequently measured in duplicate on an elemental analyzer mass spectrometer (ANCA-SL 2020, Sercon) with a precision of ±1.5 μg C and ±0.5‰, respectively. PIC was calculated as the difference between TPC and POC. Cell count samples were fixed with formalin (0.4% final concentration, buffered with hexamethylenetetramine). Cell densities were determined daily or every other day immediately after sampling using a Coulter Multisizer III. Cell-specific growth rate (μ, unit d^{-1}) was calculated as

$$\mu = (\ln N_{\text{fin}} - \ln N_0)/\Delta t \quad (3)$$

Table 1. Parameters of the seawater carbonate system. Samples for total alkalinity and DIC were taken at the beginning (t_0) and the end (t_{fin}) of the experiment. pH was calculated from alkalinity, DIC, phosphate, temperature, and salinity using the program CO2Sys (Lewis and Wallace 1998).

	Beginning of experiments (t_0)	End of experiments (t_{fin})
Total alkalinity (μeq kg^{-1})	2,572 (± 34)	2,534 (± 45)
DIC (μmol kg^{-1})	2,239 (± 49)	2,219 (± 41)
pH (total scale)	8.24 (± 0.03)	8.22 (± 0.07)

where N_0 and N_{fin} denote the cell concentrations at the beginning and the end of the experiments, respectively, and Δt is the corresponding duration of incubation in days. PIC and POC production rates were calculated from cellular inorganic and organic carbon content and cell-specific growth rates according to the following Eqs. 4 and 5.

$$\text{PIC production} = (\text{PIC/cell})\mu \quad (4)$$

$$\text{POC production} = (\text{POC/cell})\mu \quad (5)$$

To investigate the coccolith morphology in the different calcium treatments, 10 mL of culture were filtered onto cellulose–nitrate filters (Sartorius, 0.2 μm), dried, and stored in a desiccator. These samples were finally sputter-coated and examined by means of a field emission SEM.

To determine isotopic composition of DIC ($\delta^{13}C_{DIC}$), 8 mL of culture were fixed with $HgCl_2$ (140 mg final concentration), stored at 4°C, and measured on a Finnegan mass spectrometer (MAT 252) with an average precision of $\delta^{13}C = \pm 0.05‰$. The isotopic composition of CO_2 ($\delta^{13}C_{CO2}$) was calculated from $\delta^{13}C_{DIC}$ using the equation by Rau et al. (1996) on the basis of Mook et al. (1974):

$$\delta^{13}C_{CO_2} = \delta^{13}C_{DIC} + 23.644 - (9701.5/T_K) \quad (6)$$

where T_K is the absolute temperature in Kelvin. The isotopic composition is reported relative to the PeeDee belemnite standard:

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

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

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

Determination of photosynthesis, CO_2, and HCO_3^- uptake—To investigate inorganic carbon (C_i) fluxes during steady-state photosynthesis, 1000 mL of culture were concentrated by gentle filtration over a 3-μm membrane filter (Isopore, Millipore). The artificial culture media was hereby stepwise exchanged with CO_2-free artificial culture medium, buffered with 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES, 50 mmol L^{-1}, pH 8.0). The carbon flux measurements were performed with a MIMS (Isoprime; GV Instruments). The method established by Badger et al. (1994) is based on simultaneous measurements of O_2 and CO_2 during consecutive light and dark intervals. Briefly, rates of O_2 consumption in the dark and O_2 production in the light are used as direct estimates of respiration and net C_i fixation. While CO_2 uptake is calculated from the steady-state rate of CO_2 depletion at the end of the light period, the HCO_3^- uptake is derived by a mass balance equation, i.e., the difference of net C_i fixation and net CO_2 uptake. In the present study we largely followed the protocol described by Rost et al. (2006). All measurements were performed in artificial culture medium with the respective Ca concentration at 15°C. Dextran-bound sulfonamide (DBS), an inhibitor of external carbonic anhydrase, was added to the cuvette to get a final concentration of 100 μmol L^{-1}. Light and dark intervals during the assay lasted 6 min. The incident PFDs in the assays were 300 and 800 μmol photons m^{-2} s^{-1}, respectively. No C_i flux measurements could be performed for the 30 PFD treatment because the C_i fluxes were too small to create a chemical disequilibrium between CO_2 and HCO_3^- during the light phase, a prerequisite for this technique. For further details on the method and calculation we refer to Badger et al. (1994) and Rost et al. (2006). Samples for the determination of chlorophyll *a* concentration (Chl *a*) were taken after the measurements and stored at −80°C. Chl *a* was subsequently extracted in 10 mL of acetone (overnight in darkness, at 4°C) and determined with a fluorometer (Turner Designs). Chl *a* concentrations in the assay ranged from 0.18 to 0.88 μg mL^{-1}.

Results

Growth rates, PIC, and POC production—Light intensity had a strong effect on growth rates (μ), i.e., increasing rates with increasing PFDs (Fig. 1a; ANOVA, *F*-test: $p < 0.0001$). Growth rates of *E. huxleyi* ranged from 0.42 to 1.12 d^{-1}. Within the respective PFD treatment, variations in growth rates among the different Ca concentrations were small (ANOVA, *F*-test: $p > 0.05$ for 30, 800 PFD treatments; $p < 0.0001$ for 300 PFD treatments) with the exception of the 0.1 mmol L^{-1} Ca treatment (0.1 Ca treatment), in which μ was slightly reduced (0.42 d^{-1} compared with 0.5 d^{-1}).

With the exception of the 0.1 Ca treatment, the PIC production correlated positively with light intensity (Fig. 1b; ANOVA, *F*-test: $p < 0.0001$ for 1, 2.5, 10 Ca

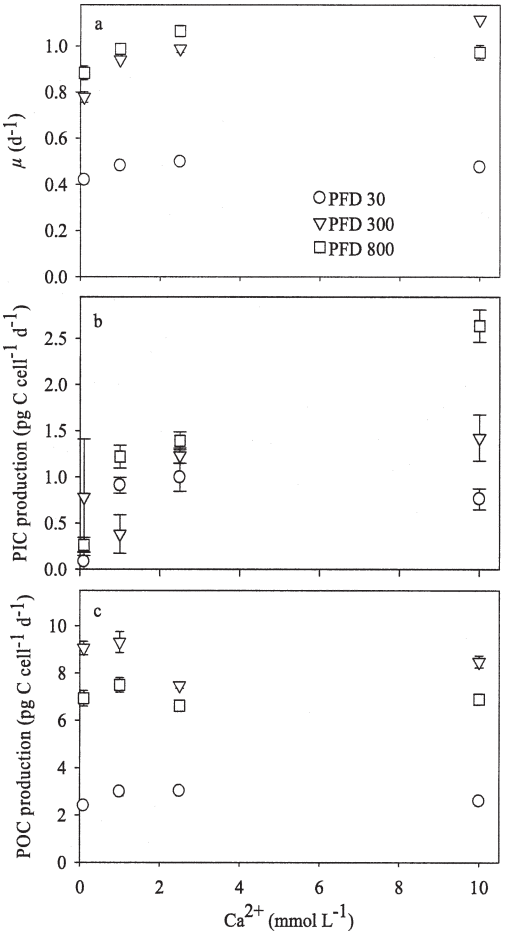

Fig. 1. Effects of Ca concentrations on (a) growth rates (μ), (b) PIC, and (c) POC production at different photon flux densities (PFDs). Symbols denote PFDs in μmol m^{-2} s^{-1}. Values represent the means of triplicate incubations (± SD).

treatments; $p > 0.05$ for 0.1 Ca treatments), and also showed a strong dependency on ambient Ca concentrations (ANOVA, *F*-test: $p < 0.0001$), whereby PIC production rates declined with decreasing Ca concentrations. Pictures taken by SEM of the 0.1 Ca treatment revealed that the cells were noncalcified; all other Ca treatments showed coccolith-bearing cells (Fig. 2). In the 1.0 Ca treatment, coccoliths and coccospheres were present, but the coccoliths displayed dissolution effects.

The POC production varied between 2.4 and 9.3 pg $cell^{-1}$ d^{-1} (Fig. 1c). At PFD 30 μmol photons m^{-2} s^{-1}, POC production rates were about 2.4 to 3.0 pg $cell^{-1}$ d^{-1} and increased by up to threefold under higher light intensities (ANOVA, *F*-test: $p < 0.0001$). The 300 PFD treatment obtained higher POC production, with values

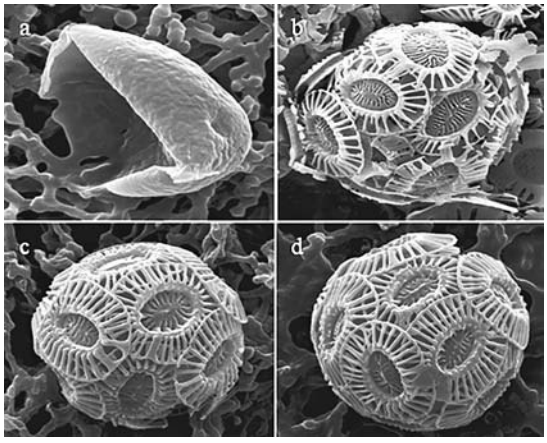

Fig. 2. Different degrees of calcification as depicted by SEM images of cells grown in artificial seawater containing (a) 0.1 mmol L^{-1}; (b) 1 mmol L^{-1}, (c) 2.5 mmol L^{-1}, and (d) 10 mmol L^{-1} Ca. Images show cells acclimated to 300 μmol photons m^{-2} s^{-1}.

between 7.5 and 9.3 pg $cell^{-1}$ d^{-1} in comparison with 800 PFD treatments, ranging from 6.6 to 7.5 pg $cell^{-1}$ d^{-1}. Within the respective light treatment, variations in POC production between the 0.1 and the 10 Ca treatments were small (ANOVA, Bonferroni's multiple comparison test: $p < 0.0001$ for 30, 300 PFD treatments; $p > 0.05$ for 800 PFD treatments). At high PFDs (300 and 800 μmol photons m^{-2} s^{-1}), POC production rates of cells acclimated to 0.1 mmol L^{-1} Ca were slightly higher compared with cells grown under 10 mmol L^{-1} Ca (ANOVA, Bonferroni's multiple comparison test: $p < 0.0001$ for 300 PFD treatments; $p > 0.05$ for 800 PFD treatments).

Photosynthesis and C_i fluxes—Net photosynthesis was measured by simultaneously monitoring O_2 and CO_2 concentrations over consecutive LD intervals. Maximum rates of photosynthesis (V_{max}) were calculated from a Michaelis–Menten fit and are shown in Fig. 3a. The 800 PFD treatments obtained higher V_{max} with values, between 740 and 900 μmol O_2 (mg Chl *a*)$^{-1}$ h^{-1}, in comparison with 300 PFD treatments ranging from 290 to 470 μmol O_2 (mg Chl *a*)$^{-1}$ h^{-1}. Over the investigated Ca range, no clear trend was observed within a respective PFD treatment (ANOVA, *F*-test: $p > 0.05$). At 300 PFD, the 10 Ca treatment had the lowest V_{max} of all Ca treatments. Acclimation to 800 PFD under different Ca concentrations caused V_{max} to be highest within the 2.5 Ca treatments and equally low at Ca concentrations of 0.1 and 10 mmol L^{-1}.

The C_i flux measurements enabled us to estimate CO_2 and HCO_3^- uptake rates following equations by Badger et al. (1994). During steady-state photosynthesis, *E. huxleyi* grown under different Ca concentrations used CO_2 as well as HCO_3^- as carbon source. Figure 3b shows the contribution of HCO_3^- uptake relative to total carbon

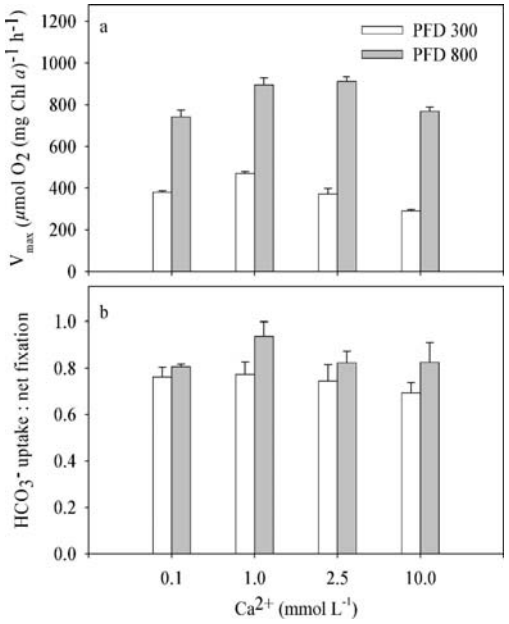

Fig. 3. (a) V_{max} and (b) ratios of HCO_3^- uptake : net photosynthesis of cells aclimated to 300 μmol photons m^{-2} s^{-1} and to 800 μmol photons m^{-2} s^{-1} as a function of Ca concentrations. V_{max} was calculated from a Michaelis–Menten fit. Ratios were based on the rates obtained at C_i concentrations of about 2 mmol L^{-1}. Error bars denote ± SD (n = 3).

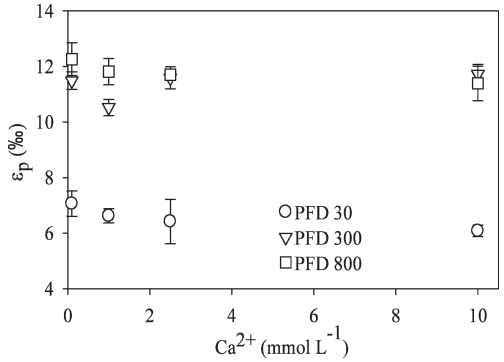

Fig. 4. Isotope fractionation (ε_p) as a function of Ca concentrations, calculated from the $^{13}C_{CO2}$ and $^{13}C_{POC}$ in the respective acclimations. Symbols denote photon flux densities (PFDs) in μmol m^{-2} s^{-1}. Values represent the means of triplicate incubations (± SD).

fixation for the conditions of the respective incubations. With values ≥0.7 the preference for HCO_3^- in *E. huxleyi* is high and further increases with light intensity. Within PFD treatments, HCO_3^- contribution was similarly high in all Ca concentrations (ANOVA, *F*-test: $p > 0.05$).

Carbon isotope fractionation—Carbon isotope fractionation showed a positive correlation with light intensity (Fig. 4; ANOVA, *F*-test: $p < 0.0001$). At high PFDs (300 and 800 μmol photons m^{-2} s^{-1}), ε_p values ranged between 10.5‰ and 12.3‰, whereas at 30 μmol photons m^{-2} s^{-1} carbon isotope fractionation was reduced to values ranging from 6.1‰ to 7.1‰. Variations in isotope fractionation with Ca concentration were small, less than 1.2‰, and showed no apparent trend over the investigated Ca range (Fig. 4; ANOVA, *F*-test: $p > 0.05$).

Discussion

The present study investigates the combined effects of varying Ca concentrations and light intensities in a calcifying strain of *E. huxleyi*. Acclimation of calcifying cells to different Ca concentrations led to different degrees of calcification within the same strain. The application of this multimethod approach provides new information on the role of calcification in *E. huxleyi*. Here we discuss the effects of varying light intensities and different Ca concentrations on calcification and photosynthesis before considering the role of calcification in photosynthetic carbon acquisition.

Growth—Cell division rates of *E. huxleyi* increased with increasing light intensity (Fig. 1a). Light-saturated growth irradiances found for *E. huxleyi* reported in Nielsen (1997) and Harris et al. (2005) are ~200 μmol photons m^{-2} s^{-1}. In accordance with these findings, we observed that cells acclimated to 300 and 800 μmol photons m^{-2} s^{-1} showed similar growth rates. This is also consistent with the previous observation that very high irradiances do not reduce growth rates in *E. huxleyi* (Nielsen 1997; Harris et al. 2005). With the exception of the 0.1 Ca treatment, Ca concentration had no effect on growth rates (Fig. 1a). Although growth rates in our study were similar to those observed in experiments that were carried out in natural seawater with the same strain of *E. huxleyi* (Rost et al. 2002; Zondervan et al. 2002), the PIC/POC ratio in the 10 Ca treatment was comparably low, with measured values of about 0.3 (Fig. 1b,c). Zondervan et al. (2002) observed PIC/POC ratios to vary between 0.3 and 1.0 under nutrient-replete conditions in this strain. We suggest that the use of artificial seawater might be a potential reason for the lower rates in our experiments, but also point out that the SEM analysis indicates normally calcified cells at high Ca concentrations.

Effect of Ca on photosynthesis—The acclimation of calcifying cells to low Ca concentrations (0.1 mmol L^{-1} in our lowest Ca concentration) did not affect their POC production (Fig. 1c), a finding that has been observed in previous investigations either by transferring decalcified cells to low Ca concentrations such as 0.1 and 1 mmol L^{-1} (Paasche 1964), by the acclimation of calcifying cells to Ca-free medium (Herfort et al. 2002), or by growing them in seawater that contains different Ca concentrations (0–

20 mmol L^{-1}; Herfort et al. 2004). These findings stand in contrast to observations by Nimer et al. (1996), who found reduced photosynthetic rates by removal of external Ca. Possible reasons for these contrasting results are difficult to assess here but may be associated with the lack of acclimation to the respective Ca concentrations in these experiments (Brownlee pers. com.) or the low temperature during centrifugation of cells (15 min at 4°C despite a growth temperature of 15°C).

Effect of Ca on calcite production—PIC production is strongly dependent on irradiance in *E. huxleyi* (e.g., Balch et al. 1992; Holligan et al. 1993; Zondervan et al. 2002). In general, light saturation irradiances for PIC production of *E. huxleyi* range from 72 to >500 μmol photons m^{-2} s^{-1} (Paasche 1964; Balch et al. 1992), which is consistent with the results of our study (Fig. 1b). PIC production of *E. huxleyi* decreased with decreasing Ca concentrations in the medium (Fig. 1b). Given the standard deviation, the PIC production can be considered to be almost nil in all 0.1 Ca treatments. The high variation in PIC production, especially under 300 μmol photons m^{-2} s^{-1}, might be due to filtration artifacts. The notion of PIC production lacking in all 0.1 Ca treatments is further supported by SEM analysis (Fig. 2a), whereas at highest Ca levels SEM analysis verified normally calcified cells.

Some response in calcification may, however, also be related to dissolution after the coccolith is extruded by the cells. Calcite dissolution can occur when the calcite saturation state (Ω) is below 1. While for the 2.5 Ca treatment Ω is greater than 1, in the 0.1 Ca treatments dissolution after calcite production is not inconceivable since Ω is 0.04. However, several lines of evidence strongly support our conclusion that reduced calcite quotas under lower Ca concentrations are the result of decreasing production rates rather than increasing dissolution. First, ^{14}C incorporation experiments by Paasche (1964) demonstrated that the low measured calcification rates under low calcium concentrations were not due to dissolution effects, but to a minimized calcification process. Even under the assumption that calcification would still occur and that every coccolith extruded would dissolve instantaneously, this has to result in a PIC content at least equivalent to the coccolith currently produced inside the coccolith vesicle. However, Paasche (1964) found that the PIC content is much lower than the equivalence of one coccolith, confirming that the low calcification rates were not due to dissolution. Moreover, SEM analysis displayed only noncalcified cells at 0.1 mmol L^{-1} Ca (Fig. 2a). If these cells were naked because of dissolution, some coccolith residues would have been visible on the filter, but this was not the case. SEM images of the 1 Ca treatment (Ω = 0.4) revealed not only that cells were fully covered with coccoliths, but also overcalcified coccospheres (images not shown) despite Ca undersaturation. This observation can be explained by the fact that coccoliths dispose of an organic membrane sheath that stabilizes the calcite against dissolution (Paasche 2001). Hence, we conclude that the cells did not calcify at 0.1 mmol Ca L^{-1} and that this finding was predominantly due to a biological effect rather than calcite dissolution.

Role of calcification under high irradiances—Various authors report that *E. huxleyi* lacks photoinhibition, even when cells are exposed to irradiances up to 1,500 μmol photons m^{-2} s^{-1} (Nielsen 1995; Nanninga and Tyrell 1996; Harris et al. 2005). To explain this unusual tolerance to high irradiance it has been proposed that calcification might serve as a metabolic protection in *E. huxleyi* when suddenly exposed to high light levels (Paasche 2001). As an energy-requiring process, calcification could provide a means to dissipate excess light energy. In the case of such a mechanism occurring under high light conditions, photoinhibition should arise when calcification ceases.

In our experiments, POC production was higher under 300 than 800 μmol photons m^{-2} s^{-1}, which indicates photoinhibition (Fig. 1c). In contrast, C_i flux assays yielded highest maximal photosynthetic rates at 800 μmol photons m^{-2} s^{-1} in all Ca treatments (Fig. 3a). These apparently conflicting results are likely to reflect the properties of the different methods applied. The C_i flux assays yield instantaneous rates of photosynthetic O_2 evolution, whereas the POC production shows integrated responses over the duration of the experiments, hence, i.e., over many days, including dark phases. Our results therefore suggest that under short-term exposure to high irradiances, *E. huxleyi* was able to maintain maximal photosynthetic rates and thus avoid photoinhibition, but over long-term exposure to high irradiances photosynthetic activity decreased.

Our results from the C_i flux assays are in agreement with various studies that observe a lack of photoinhibition under short-term exposure to high irradiances (Nielsen 1995; Nanninga and Tyrell 1996; Houdan et al. 2005). On the long-term exposure of *E. huxleyi* to high irradiances, however, only few data exist. Harris et al. (2005) showed that the POC production remained constant when cells of a noncalcifying strain of *E. huxleyi* were acclimated to high light intensities. The apparent differences from our results (Fig. 1c) may be explained by strain-specific differences, but cannot be answered conclusively here.

How, then, is the observed light-dependence of *E. huxleyi* correlated with calcification? At 800 μmol photons m^{-2} s^{-1} noncalcifying and calcifying cells showed similar POC production (Fig. 1c). Likewise, photosynthetic O_2 evolution was similar at 0.1 mmol L^{-1} and 10 mmol L^{-1} Ca (Fig. 3a). Therefore, our data do not support the hypothesis that calcification provides a means of energy dissipation under high irradiances in *E. huxleyi*. This interpretation is further supported by the observation that both low- and high-calcifying strains of *Pleurochrysis* spp. are insensitive to photoinhibition (Israel and Gonzalez 1996). Since coccospheres were lacking under 0.1 mmol L^{-1} Ca, our data further support previous studies that negated a direct role of coccoliths in photoprotection (Nanninga and Tyrell 1996; Harris et al. 2005).

Role of calcification in photosynthetic carbon acquisition—The hypothesis that photosynthesis benefits from cal-

cification by providing CO_2 is still under debate (Rost and Riebesell 2004 and references therein). Although there is increasing evidence against such a mechanism, this hypothesis seems to be widely accepted and even found its way into textbooks. We tested this theory experimentally by modifying rates of calcification and photosynthesis in *E. huxleyi* while keeping CO_2 supply constant.

Rost and Riebesell (2004) show that a calcifying and a noncalcifying strain of *E. huxleyi* are able to photosynthesize as efficiently as, or even more efficiently than, calcifying ones. Additionally, C_i flux measurements indicated that HCO_3^- uptake rates of the noncalcifying strain were even higher than in the calcifying one; thus the authors conclude that HCO_3^- utilization is not tied to calcification. Some of these findings could, however, have been associated with the different strains used. Our results were obtained from cells of the same strain and clearly show that calcification does not promote photosynthesis. Three key findings support this: (1) POC production in noncalcifying cells in comparison with calcifying cells was similar or elevated (Fig. 1c), an observation confirmed by the C_i flux assays (Fig. 3a), which yield similar or even higher maximum rates of photosynthetic O_2 evolution in noncalcifying cells. (2) According to the assumption that calcification promotes photosynthesis by providing a mechanism to access HCO_3^-, it follows that calcifying cells should have higher HCO_3^- uptake rates than noncalcifying ones. However, independent of the Ca treatments and hence the degree of calcification, the contribution of HCO_3^- uptake relative to carbon net fixation was constant (Fig. 3b). (3) Carbon isotope fractionation was used as a tool to investigate whether calcification is channeling inorganic carbon to photosynthesis. The carbon source for calcification, HCO_3^-, is about 8‰ to 10‰ enriched in ^{13}C compared with CO_2 (*see* Zeebe and Wolf-Gladrow 2001). Assuming CO_2 is produced internally via calcification (Eq. 1), it should have the same isotopic signature as the HCO_3^- previously taken up. In the case of the suggested coupling, higher fractionation values should be expected under low Ca concentrations when supposedly less HCO_3^- is used for photosynthesis. Fractionation within light levels did not, however, differ despite the large differences in Ca concentration (Fig. 4), indicating no effect of calcification on HCO_3^- use in *E. huxleyi*.

Our findings are in accordance with Paasche (1964) and Herfort et al. (2002, 2004), who performed ^{14}C incorporation experiments with *E. huxleyi*. They observed that photosynthesis in *E. huxleyi* remained constant when calcification ceased. These results bear an interesting resemblance to hermatypic corals (Gattuso et al. 2000), in which photosynthesis was also not found to be coupled to calcification. Further evidence supporting our results comes from Balch et al. (1996), who demonstrated that calcification is decoupled from photosynthesis under steady-state light-limited growth in *E. huxelyi*. Finally, the relatively high pH required for calcite precipitation represents another argument against the hypothesis that photosynthesis benefits from calcification. The pH in the coccolith vesicle of *Coccolithus pelagicus* was up to 8.3 while the pH in the cytosol was measured to be only 7.0 (Anning et al. 1996). As a consequence of this large pH difference, the coccolith vesicle would rather act as a CO_2 sink as opposed to supplying photosynthesis with additional inorganic carbon. Overall, on the basis of our data and that of previous investigations, we conclude that calcification is not involved in photosynthetic carbon acquisition of *E. huxleyi*.

In conclusion, the results of this study provide new information on the role of calcification in *E. huxleyi* and confirm findings of earlier work: (1) calcification does not provide a means of energy dissipation under high irradiances, and (2) calcification is not involved in photosynthetic carbon acquisition. These findings prompt further investigations, especially with regard to the light dependence of *E. huxleyi* and how it is affected by long- and short-term exposure to high irradiances. Furthermore, it is important to test other coccolithophore species in this respect.

The data presented here have interesting implications concerning the effects of ocean acidification on coccolithophores. It has been suggested that calcification will decrease under elevated atmospheric CO_2 levels as a result of altered carbonate chemistry (Wolf-Gladrow et al. 1999; Riebesell et al. 2000). A reduction in the degree of calcification is assumed to put coccolithophores at an ecological disadvantage, suggesting a rather "grim future" for this group of phytoplankton. However, our data demonstrate that cells perform equally well in terms of growth rate or photosynthesis despite different degrees of calcification. Therefore an understanding of calcification will be key to assessing the effects of global change on the ecological dynamics of coccolithophore communities in the future.

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Inorganic carbon acquisition in potentially toxic and non-toxic diatoms: the effect of pH-induced changes in seawater carbonate chemistry

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The effects of pH-induced changes in seawater carbonate chemistry on inorganic carbon (C_i) acquisition and domoic acid (DA) production were studied in two potentially toxic diatom species, *Pseudo-nitzschia multiseries* and *Nitzschia navis-varingica*, and the non-toxic *Stellarima stellaris*. In vivo activities of carbonic anhydrase (CA), photosynthetic O_2 evolution and CO_2 and HCO_3^- uptake rates were measured by membrane inlet MS in cells acclimated to low (7.9) and high pH (8.4 or 8.9). Species-specific differences in the mode of carbon acquisition were found. While extracellular carbonic anhydrase (eCA) activities increased with pH in *P. multiseries* and *S. stellaris*, *N. navis-varingica* exhibited low eCA activities independent of pH. Half-saturation concentrations ($K_{1/2}$) for photosynthetic O_2 evolution, which were highest in *S. stellaris* and lowest in *P. multiseries*, generally decreased with increasing pH. In terms of carbon source, all species took up both CO_2 and HCO_3^-. $K_{1/2}$ values for inorganic carbon uptake decreased with increasing pH in two species, while in *N. navis-varingica* apparent affinities did not change. While the contribution of HCO_3^- to net fixation was more than 85% in *S. stellaris*, it was about 55% in *P. multiseries* and only approximately 30% in *N. navis-varingica*. The intracellular content of DA increased in *P. multiseries* and *N. navis-varingica* with increasing pH. Based on our data, we propose a novel role for eCA acting as C_i-recycling mechanism. With regard to pH-dependence of growth, the 'HCO_3^- user' *S. stellaris* was as sensitive as the 'CO_2 user' *N. navis-varingica*. The suggested relationship between DA and carbon acquisition/C_i limitation could not be confirmed.

Introduction

Marine diatoms are key players in the ocean carbon cycle, accounting for at least 40% of the marine primary production (Nelson et al. 1995). Until recently, the effect of inorganic carbon availability on photosynthesis has been largely ignored in marine phytoplankton ecology,

Abbreviations – *a*, fractional contribution of HCO_3^- to total C_i uptake; CA, carbonic anhydrase; CCM, carbon-concentrating mechanism; C_i, inorganic carbon; 'CO_2 user', algae predominantly taking up CO_2; DA, domoic acid; DBS, dextran-bound sulphonamide (inhibitor for eCA); DIC, dissolved inorganic carbon; eCA, extracellular CA; ε_a, fractionation factor between CO_2 and HCO_3^-; ε_b, fractionation factor of HCO_3^- and CO_3^{2-}; ε_f, intrinsic fractionation factor for RubisCO; ε_p, isotope fractionation during POC formation; ε_s, equilibrium discrimination between CO_2 and HCO_3^-; 'HCO_3^- user', algae predominantly taking up HCO_3^-; iCA, intracellular CA; $K_{1/2}$, half-saturation concentration; L, leakage (CO_2 efflux : gross C_i uptake); MIMS, membrane inlet mass spectrometer; POC, particulate organic carbon; PGA, 3-phosphoglyleric acid; RubisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; V_{max}, maximum rates (substrate saturated).

particularly because dissolved inorganic carbon (DIC) is always in excess relative to other nutrients. In seawater, C_i is mainly found in the form of HCO_3^- (approximately 2 mmol l^{-1}) but also comprises low and variable concentrations of dissolved CO_2 (approximately 5–25 μmol l^{-1}). The pH, which reflects the partitioning of the carbon species, is usually about 8.2 in air-equilibrated surface waters. Elevated pH in ocean surface waters is mainly caused by intense primary production during periods with high concentrations of phytoplankton cells, e.g. towards the end of bloom periods (Hansen 2002). Intense photosynthetic activity can result in pH values as high as 9 in open marine environments (Hinga 2002) and even up to 10 in coastal lagoons and fjords (Hansen 2002). Rising atmospheric CO_2 levels caused by human-induced activities such as fossil fuel burning has affected seawater carbonate chemistry. Present day surface ocean pH is approximately 0.1 units lower than preindustrial values owing to the uptake of 'anthropogenic' CO_2 into the ocean and its subsequent dissociation resulting in an increase of the H^+ concentration. By the end of this century, it is expected that the seawater pH will have dropped by 0.4 units relative to preindustrial values (Wolf-Gladrow et al. 1999, Intergovernmental Panel on Climate Change 2007). In view of this ocean acidification as well as variations in pH during blooms, the role of inorganic carbon (C_i) acquisition has received increasing attention in phytoplankton ecology and physiology.

The enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) fixes CO_2 into organic carbon compounds. Owing to the poor affinity of RubisCO for CO_2 (K_M of 20–70 μmol l^{-1} for eukaryotic phytoplankton, Badger et al. 1998), phytoplankton cells employ carbon-concentrating mechanisms (CCMs) to increase the CO_2 concentration at the catalytic site of this enzyme. The operation of a CCM in phytoplankton cells significantly increases the efficiency of carbon fixation, thus reported apparent half-saturation concentrations ($K_{1/2}$) values for CO_2 in microalgae are <1–8 μmol l^{-1} (Raven and Johnston 1991).

Modes of CCMs have been found to vary in terms of efficiency as well as regulation between taxonomically different groups of phytoplankton (Giordano et al. 2005). This relates to the extent to which either CO_2 or HCO_3^-, or both carbon sources, are actively transported across the plasmalemma, as well as to the presence and location of carbonic anhydrase (CA). This enzyme accelerates the otherwise slow rate of conversion between HCO_3^- and CO_2. Extracellular carbonic anhydrase (eCA) activity has been suggested to be involved in HCO_3^- utilization by converting HCO_3^- to CO_2, which is subsequently taken up by cells (Sültemeyer 1998, Elzenga et al. 2000). The physiological role of intracellular carbonic anhydrase (iCA) is still not fully understood, but it is possibly involved in different processes (Badger 2003, Badger and Price 1994, Sültemeyer 1998). Another important, but often neglected, component of a CCM is the capacity of the cell to reduce the loss of accumulated C_i by minimizing the CO_2 efflux through the cell membrane (Raven and Lucas 1985, Rost et al. 2006a, 2006b).

CCMs contribute to the competitive fitness of phytoplankton species, especially under changing pH levels. While bloom-forming diatom species operate an efficient and regulated CCM that allows maintenance of high growth rates even under elevated pH (Rost et al. 2003), slow-growing diatom species may not be able to compensate for decreasing CO_2 concentrations under these conditions. Previous studies indicate that diatoms possess highly regulated and efficient CCMs with respect to changes in CO_2 supply (Burkhardt et al. 2001, Rost et al. 2003). However, whether these are general characteristics of diatoms have yet to be rigorously tested.

Some diatom species produce the neurotoxic amino acid, domoic acid (DA). Toxic diatoms cause ecological and economic problems because of the accumulation of DA in the marine food web. Understanding toxin production is complicated because both toxic and non-toxic strains of the same species co-exist, and it is currently not clear what induces DA production (Bates et al. 1998). Lundholm et al. (2004) suggested that elevated pH-triggers DA production in different strains of *Pseudo-nitzschia*. The physiological response of toxic diatoms to changes in pH is still poorly known, and to date, no study has characterized the CCMs of toxic diatoms. Furthermore, it has not yet been investigated whether the increase of intracellular content of DA is dependent on the pH-dependent changes in carbon acquisition.

The aim of the present study was to investigate the effect of pH on C_i acquisition as well as on toxin production in three marine diatom species. Cellular uptake of CO_2 and HCO_3^- during steady-state photosynthesis of the two potentially toxic species *Pseudo-nitzschia multiseries* (a bloom-forming cosmopolitan species) and *Nitzschia navis-varingica* (which occurs in coastal marine areas and in marine ponds) as well as the non-toxic *Stellarima stellaris* (a widely distributed species) was estimated by membrane inlet mass spectrometric (MIMS) measurements. This approach was used to determine substrate preferences for CO_2 and HCO_3^- as well as possible shifts in carbon source and cellular leakage (CO_2 efflux/gross C_i uptake) with changing pH. To further characterize the CCM of each species, measurements of iCA and eCA activities were performed by monitoring ^{18}O exchange from doubly labelled $^{13}C^{18}O_2$.

Materials and methods

Culture and experimental conditions

Stellarima stellaris (isolate from the Sound, Denmark), *N. navis-varingica* (VHL987) and *P. multiseries* (OKPm013-2) were grown in dilute batch cultures in sterile-filtered (0.2 μm) unbuffered seawater, enriched with nutrients, trace metals and vitamins according to f/2 medium (Guillard and Ryther 1962). Silicate was added to a concentration of 317 μmol l^{-1}. Experiments were carried out using a light:dark cycle of 16:8 h at a constant temperature of 15°C. A photon flux density of 200 μmol photons m^{-2} s^{-1} was applied using daylight fluorescence lamps that provided a spectrum similar to that of sunlight. The applied photon flux density ensured that neither growth nor production of DA was light limited (Bates et al. 1998). Each treatment was incubated in triplicate in sterile 2.4-l borosilicate bottles. Gentle rotation of the culture flasks five times a day ensured that the cells were kept in suspension.

The medium used for the experiments was adjusted by addition of HCl or NaOH to low pH (7.9) and high pH (8.4 for *S. stellaris* and *P. multiseries*, and 8.9 for *N. navis-varingica*) on the National Bureau of Standards (NBS) scale. This corresponds to CO_2 concentrations of 31, 9.2 and 2.2 μmol kg^{-1}, respectively (Table 1). pH was measured using a pH/ion meter (model pMX 3000/pH; WTW, Weilheim, Germany) that was calibrated (two-point calibration) on a daily basis. Daily or twice-daily dilutions with fresh media ensured that the pH level remained constant (±0.05 units) and that the cells stayed in the mid-exponential growth phase. High pH levels were selected based on the upper pH limits observed in the selected species, ensuring that growth rates were not affected by the pH levels chosen. Growth rates were about 0.70 day^{-1} in *S. stellaris*, 0.95 day^{-1} in *P. multiseries* and 0.59 day^{-1} in *N. navis-varingica*. Cell concentrations ranged between 500 and 3000 cells ml^{-1} for the three species.

Determination of seawater carbonate chemistry

Alkalinity samples were taken from the filtrate (Whatman GFF filter; approximately 0.6 μm) and stored in 300-ml borosilicate flasks at 4°C and measured in triplicate by potentiometric titration with an average precision of 8 μEq kg^{-1} (Brewer et al. 1986). Total alkalinity was calculated from linear Gran Plots (Gran 1952). DIC samples were sterile-filtered (0.2 μm) and stored in 13-ml borosilicate flasks free of air bubbles at 4°C until they were measured with a total carbon analyzer (Shimadzu TOC-5050A 's-Hertogenbosch, The Netherlands) with an average precision of 17 μmol kg^{-1} in triplicate.

The carbonate system was calculated from alkalinity, DIC, silicate, phosphate, temperature and salinity using the CO2Sys program (Lewis and Wallace 1998). Equilibrium constants of Mehrbach et al. (1973) refitted by Dickson and Millero (1987) were used. The carbonate chemistry was kept constant over the duration of the experiment (Table 1).

Table 1. Parameters of the seawater carbonate system were calculated from DIC, pH, silicate, phosphate, temperature and salinity using the CO2Sys program (Lewis and Wallace 1998). Errors denote ± SD (n = 3).

pH (NBS)	TA (μEq kg^{-1})	DIC (μmol kg^{-1})	CO_2 (μmol kg^{-1})	pCO_2 (Pa)
7.9 (±0.05)	2351 ± 82	2213 ± 80	31 ± 1.1	*82.8 ± 3.0*
8.4 (±0.05)	2651 ± 94	2240 ± 84	9.2 ± 0.3	*24.6 ± 0.9*
8.9 (±0.05)	*3115 ± 78*	*2171 ± 60*	*2.2 ± 0.1*	*6.0 ± 0.2*

Sampling

After acclimation for at least 7 days, cells were harvested by gentle filtration over an 8-μm membrane filter (Isopore; Millipore, Schwalbach/Ts., Germany). Subsequently, the cells were washed with CO_2-free f/2 medium buffered with 50 mmol l^{-1} HEPES (pH 8.0). The samples were then used for determination of carbon (C_i) fluxes and CA activities with the MIMS. Samples for determination of Chl *a* concentration were taken after the measurements and stored at −80°C. Chl *a* was subsequently extracted in 10 ml acetone (overnight in darkness at 4°C) and determined with a Turner Designs Fluorometer.

Determination of CA activity

Activity of eCA and iCA was determined by measuring the loss of ^{18}O from doubly labelled $^{13}C^{18}O_2$ to water caused by the interconversion of CO_2 and HCO_3^- (Silverman 1982). The determination of CA activity was performed with a sector field multicollector mass spectrometer (Isoprime; GV Instruments, Manchester, UK) through a gas-permeable polytetrafluoroethylene membrane (PTFE, 0.01 mm) inlet system. The reaction sequence of ^{18}O loss from initial $^{13}C^{18}O^{18}O$ (m/z = 49), through the intermediate $^{13}C^{18}O^{16}O$ (m/z = 47) to the final isotopomer $^{13}C^{16}O^{16}O$ (m/z = 45) was recorded continuously. The ^{18}O enrichment was calculated as:

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

CA measurements were performed in 8 ml f/2 medium buffered with 50 mmol l^{-1} HEPES (pH 8.0) at 15°C. To avoid interference with light-dependent C_i uptake by the cells, all measurements were carried out in the dark. When chemical equilibrium was reached after injection of 1 mmol l^{-1} $NaH^{13}C^{18}O$, the uncatalysed ^{18}O loss was monitored for about 8 min prior to the addition of cells. eCA activity was calculated from the increasing rate of ^{18}O depletion after addition of the cells (slope S_2) in comparison with the uncatalysed reaction (slope S_1) and normalized on a Chl *a* basis (Badger and Price 1989):

$$U = \frac{(S_2 - S_1)\times 100}{S_1 \times \mu g\ Chl\ a} \quad (2)$$

iCA activity was determined in the presence of 100 μmol l^{-1} dextran-bound sulphonamide (DBS), an inhibitor of eCA. The drop in the log(enrichment) was calculated by extrapolation of S_2 back to the time of cell injection [Δ as defined by Palmqvist et al. (1994)]. Values of Δ are expressed in arbitrary units per μg Chl *a*. Chl *a* concentrations in CA assays ranged from 0.13 to 1.34 μg ml^{-1}.

Determination of net photosynthesis, CO_2 and HCO_3^- uptake

C_i fluxes were determined during steady-state photosynthesis with the same MIMS as for CA measurements. The method established by Badger et al. (1994) is based on simultaneous measurements of O_2 and CO_2 during consecutive light and dark intervals. Known amounts of C_i were added to measure photosynthesis and carbon uptake rates as a function of CO_2 and HCO_3^- or DIC concentrations. Net photosynthesis, CO_2 uptake and HCO_3^- uptake were calculated according to the equations of Badger et al. (1994). All measurements were performed in initially CO_2-free f/2 medium buffered with 50 mmol l^{-1} HEPES (pH 8.0) at 15°C. DBS was added to the cuvette to a final concentration of 100 μmol l^{-1}. Light and dark intervals during the assay lasted 6 min. The incident photon flux density was 300 μmol photons m^{-2} s^{-1}. Further details on the method and calculations are given in Badger et al. (1994) and Rost et al. (2007). Chl *a* concentrations in the assay ranged from 0.28 to 1.57 μg ml^{-1}.

Carbon isotope fractionation

Samples for particulate organic carbon (POC) were filtered onto precombusted (500°C, 12 h) GFF filters (approximately 0.6 μm) and stored in precombusted (500°C, 12 h) Petri dishes at −20°C. Prior to analysis, POC filters were treated with 200 μl HCl (0.1 N) to remove all inorganic carbon. POC and related $\delta^{13}C$ values were subsequently measured in duplicate on an EA mass spectrometer (ANCA-SL 2020; 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:

$$\delta^{13}C_{Sample} = \left[\frac{(^{13}C/^{12}C)_{Sample}}{(^{13}C/^{12}C)_{PDB}} - 1\right]\times 1000 \quad (3)$$

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

$$\varepsilon_p = \frac{\delta^{13}C_{CO_2} - \delta^{13}C_{POC}}{1 + \frac{\delta^{13}C_{POC}}{1000}} \quad (4)$$

To determine isotopic composition of DIC ($\delta^{13}C_{DIC}$), samples were sterile filtered (0.2 μm), fixed with $HgCl_2$ (approximately 140 mg l^{-1} final concentration) and stored at 4°C. Measurements of $\delta^{13}C_{DIC}$ were performed with a Finnegan mass spectrometer (MAT 252) at a precision of $\delta^{13}C = \pm 0.05‰$. The isotopic composition of CO_2 ($\delta^{13}C_{CO2}$) was calculated from $\delta^{13}C_{DIC}$, making use of a mass balance relation (Zeebe and Wolf-Gladrow 2001):

$$\delta^{13}C_{HCO_3^-} = \frac{\delta^{13}C_{DIC}[DIC] - (\varepsilon_a[CO_2] + \varepsilon_b[CO_3^{2-}])}{(1 + \varepsilon_a\times 10^{-3})[CO_2] + [HCO_3^-] + (1 + \varepsilon_b\times 10^{-3})[CO_3^{2-}]} \quad (5)$$

$$\delta^{13}C_{CO_2} = \delta^{13}C_{HCO_3^-}(1 + \varepsilon_a\times 10^{-3}) + \varepsilon_a \quad (6)$$

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

Toxin analyses

The samples for toxin analyses (600 or 1000 ml) were filtered through a 3-μm polycarbonate filter, which was never allowed to dry out. The residue on the filter was rinsed with fresh culture medium and subsequently transferred to a falcon tube and adjusted to a final volume

of 4 ml. Initial measurements on the filtered medium showed no DA (i.e. extracellular DA) in any of the treatments and consequently filtered medium was not subsequently measured. Cell counts were used for determination of toxin content per cell. Until further analysis, samples were stored at −20°C.

For preparation of the samples, 4 ml of frozen material were thawed and subsequently centrifuged at 4°C for 15 min at 2100 *g*. Because diatoms partially break during freezing and thawing cycles and cell content leaks into the medium, DA had to be determined in cell pellets and supernatant. Five hundred microlitres of the supernatant was centrifuged (Eppendorf 5415 R; Eppendorf, Hamburg, Germany) for 30 s at 800 *g* through a spin filter (pore size 0.45 μm, Millipore-Ultrafree, Eschborn, Germany) and frozen at −20°C until LC-MS/MS analysis for measurement of DA in the cell-free fraction. Cell pellets were resuspended in 0.5 ml water–methanol (1:1 v/v), transferred to FastPrep tubes containing 0.9 g of lysing matrix D (Thermo Savant, Illkirch, France) and subsequently homogenized by reciprocal shaking at maximum speed for 45 s in a Bio101 FastPrep instrument (Thermo Savant). After homogenization, samples were centrifuged at 16 100 *g* at 4°C for 15 min. Supernatants were removed and centrifuged for 30 s at 800 *g* through a spin filter and frozen at −20°C until LC-MS/MS analysis of DA in the cellular fraction.

Mass spectrometric measurements were performed on an ABI-SCIEX-4000 Q Trap, triple quadrupole mass spectrometer equipped with a TurboSpray® interface coupled to an Agilent model 1100 LC. Analyses for DA were performed in triplicate. The analytical column (250 × 4.6 mm) was packed with 5 μm Luna C18 (Phenomenex, Aschaffenburg, Germany) and maintained at 25°C. The flow rate was 1.0 ml min^{-1} and gradient elution was performed with two eluents, where eluent A was 2 mmol l^{-1} ammonium formate and 50 mmol l^{-1} formic acid in water and eluent B was 2 mmol l^{-1} ammonium formate and 50 mmol l^{-1} formic acid in acetonitrile/water (95:5 v/v). The gradient was as follows: 9 min column equilibration with 87% A, 2 min isocratic with 87% A, then linear gradient until 10 min to 50% A, then until 11 min return to initial conditions 87% A. Total run time was 20 min. Five microlitres of sample were injected.

Measurements were carried out in the multiple reaction monitoring (MRM) mode by selecting the following transitions for DA (precursor ion>fragment ion): m/z 312>266 (quantifier) and m/z 312>161 (qualifier). Dwell times of 150 ms were used for each transition. For these studies, the following source parameters were used – curtain gas: 25 psi, temperature: 600°C, ion-spray voltage: 5500 V, nebulizer gas: 55 psi, auxiliary gas: 70 psi, interface heater: on, and declustering potential: 66 V.

Results

Photosynthesis and C_i fluxes

During steady-state photosynthesis, CO_2 and HCO_3^- were taken up simultaneously by all species (Table 2). Maximum rates (V_{max}) and half-saturation concentrations ($K_{1/2}$) were obtained from a Michaelis–Menten fit to the data and are summarized in Table 2. The acclimation of a species to low and high pH had no effect on the V_{max} of photosynthetic O_2 evolution in the assay, i.e. V_{max} remained constant (Table 2). In comparison, *P. multiseries* exhibited the highest photosynthetic rates on a Chl *a* basis. In all investigated species, $K_{1/2}$ values for photosynthetic O_2 evolution decreased with rising pH (Table 2). Highest $K_{1/2}$ values were recorded in *S. stellaris* and lowest in *P. multiseries*. In all three species, $K_{1/2}$ (CO_2) values for photosynthesis were about one order of

Table 2. $K_{1/2}$ and V_{max} of photosynthesis, net CO_2 uptake and HCO_3^- uptake for *Stellarima stellaris*, *Pseudo-nitzschia multiseries* and *Nitzschia navis-varingica* acclimated to low and high pH. Kinetic parameters were calculated from a Michaelis–Menten fit to the combined data of several independent measurements. Values for $K_{1/2}$ and V_{max} are given in μmol l^{-1} and μmol (mg Chl *a*)$^{-1}$ h^{-1}, respectively. Error bars denote ±SD.

	Photosynthesis				Net CO_2 uptake		HCO_3^- uptake	
pH (NBS)	$K_{1/2}$ (CO_2)	V_{max} (CO_2)	$K_{1/2}$ (DIC)	V_{max} (DIC)	$K_{1/2}$ (CO_2)	V_{max} (CO_2)	$K_{1/2}$ (HCO_3^-)	V_{max} (HCO_3^-)
S. stellaris								
7.9	7.4 ± 1.7	258 ± 17	572 ± 133	258 ± 17	—	40 ± 29	329 ± 150	219 ± 23
8.4	4.0 ± 0.5	262 ± 9	304 ± 41	264 ± 10	—	8.6 ± 5.3	227 ± 34	254 ± 9
P. multiseries								
7.9	3.5 ± 0.5	363 ± 20	327 ± 57	368 ± 22	4.6 ± 0.6	175 ± 10	241 ± 49	190 ± 14
8.4	2.2 ± 0.3	354 ± 15	223 ± 39	369 ± 18	2.3 ± 0.3	148 ± 8	186 ± 63	218 ± 15
N. navis-varingica								
7.9	4.6 ± 0.7	262 ± 10	494 ± 67	261 ± 9	6.0 ± 1.0	221 ± 10	—	42 ± 4
8.9	3.1 ± 0.9	287 ± 19	301 ± 84	290 ± 18	6.6 ± 1.2	203 ± 11	—	89 ± 8

magnitude lower than the K_M (CO_2) values known for RubisCO in diatoms (approximately 30–40 μmol CO_2 l^{-1}; Badger et al. 1998).

In terms of CO_2 uptake, the $K_{1/2}$ values for *N. navis-varingica* and *P. multiseries* were generally between 4 and 7 μmol l^{-1}, only in *P. multiseries* the acclimation to high pH induced higher affinities (Table 2). The V_{max} for net CO_2 uptake for *N. navis-varingica* and *P. multiseries* ranged between 148 and 221 μmol (mg Chl a)$^{-1}$ h^{-1}. In *S. stellaris*, net CO_2 uptake was very low and hence no $K_{1/2}$ values could be estimated. Regarding the HCO_3^- uptake, $K_{1/2}$ values in *P. multiseries* and in *S. stellaris* were generally <330 μmol l^{-1} and decreased when acclimated to high pH. In *S. stellaris*, the V_{max} of HCO_3^- uptake was slightly higher than that for *P. multiseries*. For *N. navis-varingica*, the HCO_3^- uptake was low and could not be described by Michaelis–Menten kinetics.

Using the uptake kinetics obtained in the assay, the contribution of HCO_3^- uptake relative to carbon fixation for the conditions of the respective incubations was estimated (Fig. 1). The preference for HCO_3^- uptake strongly increased in *N. navis-varingica* with rising pH, while the different pH acclimations yielded similar ratios in *P. multiseries* and *S. stellaris*. With values larger than 85%, the preference for HCO_3^- is very high in *S. stellaris*, whereas *P. multiseries* reached values of approximately 55%. In *N. navis-varingica*, the contribution of HCO_3^- uptake relative to net fixation accounted for at most approximately 30% under high pH, reflecting a strong preference for CO_2.

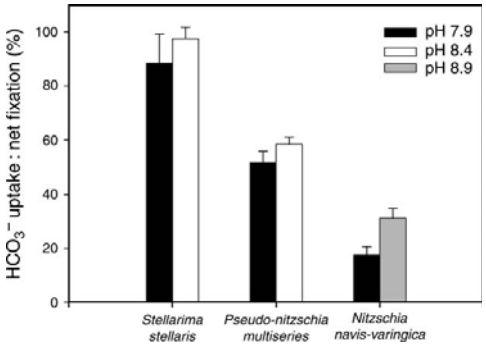

Fig. 1. Ratios of HCO_3^- uptake to net photosynthesis of cells acclimated to different pH levels. Ratios from MIMS measurements were based on the rates obtained at C_i concentrations of about 2 mmol l^{-1}.

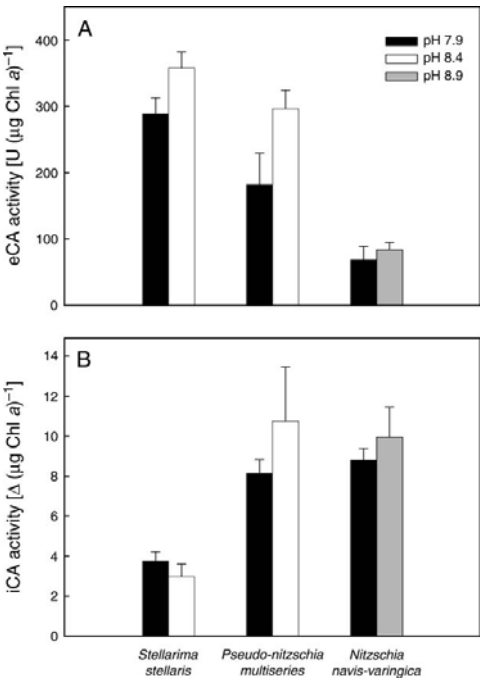

Fig. 2. Chl *a*-specific activities of (A) eCA and (B) iCA activities from cells acclimated to low and high pH. Values represent the means of triplicate incubations (±SD).

eCA and iCA activity

Activity of eCA was high in *P. multiseries* and *S. stellaris* and increased with elevated pH (Fig. 2A). In *N. navis-varingica*, eCA activities were low and remained constant independent of the pH. The activities of iCA remained constant in all investigated species, independent of the pH of the acclimation (Fig. 2B).

Carbon isotope fractionation and leakage

Carbon isotope fractionation decreased with rising pH in all investigated species (Fig. 3). The strongest reduction in ε_p was observed in *N. navis-varingica*, with ε_p being 9.8‰ under low pH and 3.7‰ at high pH. *S. stellaris* obtained highest fractionation values in comparison with the two other species, its ε_p ranged between 11.5 and 14.4‰.

The leakage of cells, i.e. the proportion of C_i efflux compared with gross C_i uptake, was estimated by MIMS through the CO_2 efflux recorded immediately after the light had been turned off (Badger et al. 1994). For all species, leakage was highest at the lowest CO_2 concentrations and levelled off towards higher CO_2 concentrations in the assay (Fig. 4). With increasing pH, leakage appeared to decrease mainly in *N. navis-varingica*.

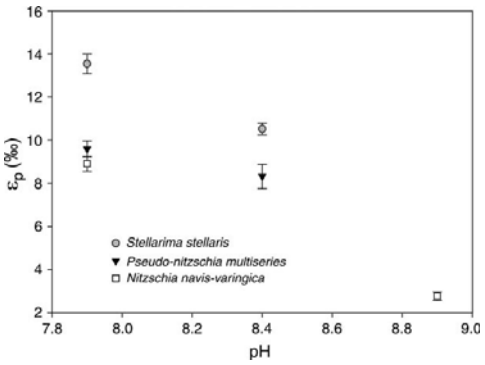

Fig. 3. Isotope fractionation (ε_p) as a function of pH, calculated from the $^{13}C_{CO2}$ and $^{13}C_{POC}$ in the respective acclimations of each species. Error bars denote ±SD (n = 3).

DA production

The content of DA per cell differed with pH, both for *P. multiseries* and for *N. navis-varingica*. Increasing pH resulted in increasing content of DA (Table 3). The differences were, however, only significant between the pH levels for *P. multiseries* (Student's *t*-test, $P < 0.01$). No DA was detected in *S. stellaris* at any of the pH levels.

Discussion

The application of MIMS techniques such as the estimation of CA activities and C_i fluxes, in combination with analyses of ^{13}C fractionation, allowed detailed characterization of the CCM in each species. Acclimations to low and high pH were performed in unbuffered seawater with low cell densities to simulate natural conditions as closely as possible. High affinities for C_i were observed in all investigated species, indicating the operation of a CCM (Table 2). All species upregulated their CCM activity when acclimated to high pH. Despite these similarities, strong species-specific differences in the modes of carbon acquisition existed.

Photosynthetic O_2 evolution

Apparent $K_{1/2}(CO_2)$ for photosynthesis lower than K_M (CO_2) of RubisCO provides evidence for the operation of a CCM (Badger et al. 1998). In our experiments, $K_{1/2}(CO_2)$ values for photosynthesis were low in all investigated species, ranging from 2.2 to 7.4 μmol CO_2 (Table 2). These values are about an order of magnitude lower than known K_M (CO_2) values for RubisCO in diatoms (31–36 μmol CO_2 l^{-1} in two strains of *Cylindrotheca* sp.

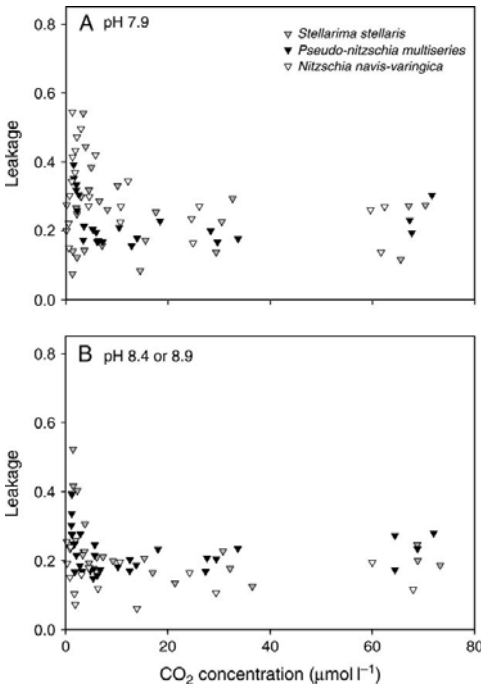

Fig. 4. Leakage of the cells, i.e. the proportion of C_i efflux relative to gross C_i uptake, as a function of the CO_2 concentration in the C_i flux assay from each species acclimated to (A) low and (B) high pH.

and 41 μmol CO_2 l^{-1} in *Phaeodactylum tricornutum*; Badger et al. 1998), indicating that the investigated species possessed highly efficient CCMs. All three species had upregulated their carbon acquisition with decreasing CO_2 supply (Table 2). The observed affinities are comparable with previous findings obtained with MIMS techniques in marine diatoms (Burkhardt et al. 2001, Rost et al. 2003). By measuring the photosynthetic O_2 evolution upon the addition of varying C_i concentrations and assuming equilibrium concentrations for CO_2,

Table 3. The intracellular content of DA in the investigated diatoms acclimated to different pH levels. Values represent the mean of triplicates ± SD.

	Nitzschia navis-varingica		*Pseudo-nitzschia multiseries*		
pH	7.9	8.9	7.9	8.4	8.9
Intracellular toxin content (pg $cell^{-1}$)	158 ± 15	213 ± 28	1.9 ± 0.3	4.2 ± 0.4	140 ± 8

$K_{1/2}(CO_2)$ values for photosynthetic O_2 evolution have been estimated (Burns and Beardall 1987, Colman and Rotatore 1995, Mitchell and Beardall 1996). Mitchell and Beardall (1996) calculated the $K_{1/2}(CO_2)$ value to be approximately 1.09 μmol l^{-1} at pH 7.5 in the sea-ice diatom *Nitzschia frigida*. Colman and Rotatore (1995) demonstrated $K_{1/2}(CO_2)$ values of 1.44 μmol l^{-1} for *Cyclotella* sp. and 4.01 μmol l^{-1} for *P. tricornutum*. For the latter species, Burns and Beardall (1987) obtained an even lower $K_{1/2}(CO_2)$ value of 0.53 μmol CO_2 l^{-1}. In summary, the CO_2/pH-induced changes in apparent C_i affinities and the generally low $K_{1/2}(CO_2)$ for photosynthesis indicate highly efficient and regulated CCMs in the investigated species.

Carbon sources and uptake kinetics

To understand why a certain species appears to have a more or less efficient carbon acquisition, in other words different abilities to reach C_i-saturated rates in photosynthesis, the different components of the CCM must be characterized. In the present study, CO_2 and HCO_3^- rates were estimated following the method of Badger et al. (1994). This approach has the advantage that it also allows determination of carbon uptake kinetics during steady-state photosynthesis. While several studies demonstrated that simultaneous uptake of CO_2 and HCO_3^- occurs in marine diatoms (e.g. Burns and Beardall 1987, Korb et al. 1997, Rotatore et al. 1995, Tortell and Morel 2002), a finding that is consistent with our results, little information exists on uptake kinetics for individual carbon sources (e.g. Burkhardt et al. 2001, Rost et al. 2003, Rost et al. 2006a).

The efficiency of a CCM is strongly depending on the characteristics of the C_i uptake systems. Higher transport rates for CO_2 and/or HCO_3^- can be achieved in two ways: either by increasing the affinities for the respective C_i species through a higher substrate-binding capacity (e.g. Amoroso et al. 1998, Matsuda and Colman 1995, Palmqvist et al. 1994) or by an increase in the number of transporters (e.g. Burkhardt et al. 2001, Rost et al. 2003). According to our results, species responded differently to pH-induced changes in carbonate chemistry. *P. multiseries* used CO_2 and HCO_3^- in equal quantities, which did not change with pH (Fig. 1). This is the result of an increased substrate affinity of the CO_2 uptake system, which compensated for the lower CO_2 availability at high pH (Fig. 1). *N. navis-varingica* was characterized by a strong preference for CO_2 ('CO_2 user'), although the contribution of HCO_3^- uptake relative to net fixation increased with rising pH (Table 2). The latter might be because of a larger number of HCO_3^- transporters when acclimated to high pH (Table 2). *S. stellaris* showed a strong preference for HCO_3^- ('HCO_3^- user') irrespective of the acclimation pH (Fig. 1). This observation is consistent with constitutively expressed C_i transport systems (Table 2). The large differences in preference for C_i sources in the group of diatoms are in agreement with those of Burkhardt et al. (2001) who showed that *Thalassiosira weissflogii* exhibited a much higher proportion of HCO_3^- uptake relative to total C_i uptake compared with *P. tricornutum*, the latter preferring CO_2 even at the highest pH level. Rost et al. (2003) showed an increasing contribution of HCO_3^- to total C_i uptake with decreasing CO_2 concentrations as a consequence of both an increasing number of HCO_3^- transport components and the induction of high-affinity C_i uptake systems. Hence, based on our data and on previous investigations, marine diatoms appear to strongly differ in terms of their preferred C_i source and the regulation of its uptake.

CA activity and its dependence on the carbon source

The enzyme CA plays an important role in carbon acquisition by accelerating the otherwise slow interconversion of CO_2 and HCO_3^-, both inside the cell and at the cell surface. The activities of eCA strongly differed between species in our investigation (Fig. 2A), being highest with 360 U (μg Chl *a*)$^{-1}$ in *S. stellaris* and lowest with 80 U (μg Chl *a*)$^{-1}$ in *N. navis-varingica*. In other words, these values correspond to an enhancement in the interconversion between HCO_3^- and CO_2 relative to the uncatalysed rate by 360 and 80% per μg Chl *a*, respectively. These activities are within the same range as those reported in Burkhardt et al. (2001) and Rost et al. (2003) [note that activities in these studies were erroneously stated as (mg Chl *a*)$^{-1}$ instead of (μg Chl *a*)$^{-1}$]. Mitchell and Beardall (1996) used a potentiometric approach to estimate CA activities (Wilbur and Anderson 1948) in *N. frigida*. They measured low eCA activities of 0.123 (μg Chl *a*)$^{-1}$ Wilbur–Anderson units, which corresponds to an enhancement in the rate constants of only approximately 12% per μg Chl *a* relative to the uncatalysed rate. According to our results and those of Mitchell and Beardall (1996), we conclude that eCA plays only a minor role in carbon acquisition in the genus *Nitzschia*.

The induction of eCA activity was found to be pH dependent both in laboratory culture experiments (Sültemeyer 1998, Burkhardt et al. 2001, Badger 2003, Rost et al. 2003) and in field experiments (Berman-Frank et al. 1994, Tortell et al. 2006). It is a common notion that eCA is involved in *indirect* HCO_3^- utilization by converting HCO_3^- to CO_2, which could then be actively

transported through the plasma membrane and subsequently used for photosynthesis (Elzenga et al. 2000, Sültemeyer 1998, Tortell et al. 2006). It should be emphasized here that high eCA activities would provide an advantage especially for large cells because large phytoplankton are more prone to CO_2 shortage in their diffusive boundary layer (Wolf-Gladrow and Riebesell 1997). Such a function of eCA in supplying CO_2 from the large HCO_3^- pool would, however, not apply at high pH because the equilibrium is strongly on the side of HCO_3^-.

In the present study, the investigated diatoms are rather large with volumes of 1030 μm^3 for *P. multiseries*, 4350 μm^3 for *N. navis-varingica* and 7720 μm^3 for *S. stellaris*. Although the highest level of eCA activity was found under high pH in the largest diatom species *S. stellaris* (Fig. 2A), this species showed a strong preference for HCO_3^- uptake and *not* for CO_2 uptake (Fig. 1). Hence, the common notion that eCA supplies CO_2 to the uptake systems is called into question in this situation. Here, we propose a mechanism that acts as a C_i-recycling mechanism at high pH in 'HCO_3^- user' (Fig. 5A), i.e. CO_2 leaking out of the cell would be converted by eCA to HCO_3^- and subsequently taken up through HCO_3^- transporters. Such a mechanism would be most efficient when the CA-mediated conversion is localized to the periplasmic space, i.e. in close vicinity to the HCO_3^- transporter. It can be hypothesized that the HCO_3^- transport process is linked to the eCA activity. Support for this idea comes from red blood cells where a plasma membrance HCO_3^-/Cl^- transporter physically binds a CAII protein (Sterling et al. 2001). In contrast to 'HCO_3^- users' like *S. stellaris*, the CO_2 user *N. navis-varingica* was characterized by low levels of eCA even under high pH (Fig. 2A). Because of rather low eCA activities, CO_2 leaking out of the cell is prevented from fast conversion to HCO_3^-, and a disequilibrium at the cell surface persists (Fig. 5B). Elevated CO_2 in turn increases the probability that the CO_2 is transported back into the cell through CO_2 transport systems. We therefore suggest that low or absent eCA activities in CO_2 users allow for efficient CO_2 recycling at high pH (Fig. 5B).

The proposed C_i-recycling mechanisms for CO_2 and HCO_3^- users at elevated pH would explain results from previous studies, i.e. the correlation of high eCA activity and HCO_3^- uptake and the lack of eCA in CO_2 users. Burkhardt et al. (2001) observed high eCA activities and high HCO_3^- uptake rates in *T. weissflogii*, while *P. tricornutum* showed a preference for CO_2 and eCA activities were low. *Skeletonema costatum* combined both extremes, i.e. high eCA activities and predominant HCO_3^- uptake under high pH, whereas eCA was lacking at low pH accompanied by a strong preference for CO_2 (Rost et al. 2003). We consequently conclude, based on our data and that of previous investigations, that the presence or absence of eCA allows for a more efficient C_i recycling in 'HCO_3^-' and 'CO_2' users, respectively.

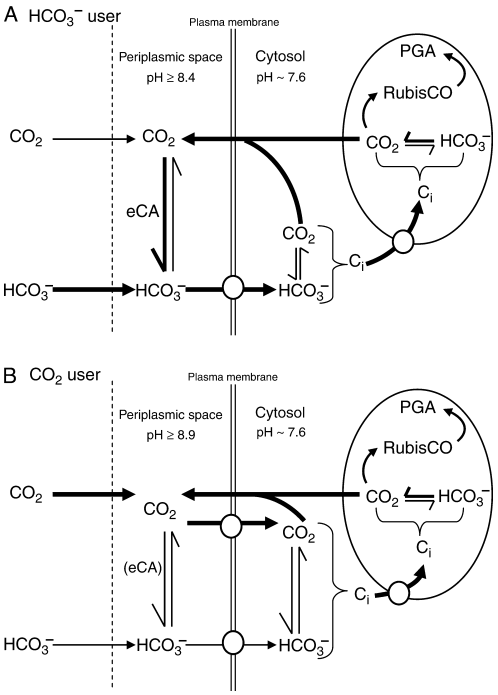

Fig. 5. Model of the C_i-recycling mechanism. The cytosolic pH is assumed to be approximately 7.6 (Burns and Beardall 1987, Colman and Rotatore 1995) while the pH in the periplasmic space is assumed to be elevated with respect to the pH in the bulk media. (A) In 'HCO_3^- user' as *Stellarima stellaris*, the CO_2 leaking out of the cell would be converted by eCA to HCO_3^- and subsequently taken up through HCO_3^- transporters. (B) In 'CO_2 user' as *Nitzschia navis-varingica*, CO_2 is prevented from conversion to HCO_3^- and a disequilibrium in the boundary layer persists. As the CO_2 leaking out of the cell remains in the form of CO_2 at the cell surface, the probability increases that the CO_2 is transported back into the cell through CO_2 transport systems. Note that in the chloroplast CO_2 is being fixed by RubisCO to produced PGA and sugars.

The physiological role of internally located CA (iCA) is still poorly understood (Sültemeyer 1998) because of the occurrence of multiple CA forms in the cytosol, chloroplast and mitochondria, but there is evidence that iCAs are important components of the CCM (Badger and Price 1994, Sültemeyer 1998, Badger 2003). Prior to our discussion on iCA activities, we would like to point out some of the assumptions underlying the applied approach to estimate iCA activities (Palmqvist et al. 1994). First, it should be noted that the results shown in Fig. 2B do not

differentiate between the multiple CA forms and their location. Estimates on iCA activities are also dependent on the rate of diffusive influx of ^{18}O-labelled CO_2 and thus can be altered by the diffusive properties of the cell membrane, intracellular pH and cell size and shape. In addition, Rost et al. (2003) showed that assessing iCA activities without applying inhibitors for eCA affects the absolute estimates. Because of these uncertainties, the following species comparison should be interpreted with caution.

High iCA levels were expressed in the CO_2 user *N. navis-varingica*, while lowest activities were found in the HCO_3^- user *S. stellaris* (Fig. 2B). Provided that iCA observed in the latter species is predominantly reflecting CA located in the cytosol, the low activity would prevent the taken up HCO_3^- from being converted to CO_2 and hence it would remain in the form of HCO_3^- for which cell membranes are highly impermeable. In contrast, high iCA levels in the CO_2 user *N. navis-varingica* enhance the conversion of CO_2 to HCO_3^- in the cytosol. This interpretation of our data is supported by the observation of Price and Badger (1989) that the absence of cytosolic CA activity in *Synechococcus*, a HCO_3^- user, is crucial for minimizing CO_2 leakage. Consequently, the expression of human CA in the cytosol led to loss of the ability to accumulate internal C_i. All data together, we propose that the presence or absence of iCA functions to minimize the loss of CO_2. Evidence for such a CO_2-trapping mechanism is supported by a model study at the level of the chloroplast (Thoms et al. 2001). Further research in marine diatoms is needed to evaluate the potential role of iCA acting as a CO_2-trapping mechanism.

Carbon isotope fractionation and leakage

Carbon isotope fractionation can provide information on modes of carbon uptake in marine phytoplankton (Raven and Johnston 1991, Rost et al. 2002). The interpretation of such data often remains complicated because of a lack of knowledge on processes involved in fractionation. In the present study, the information on the CCM derived by MIMS techniques permits a more thorough analysis of carbon isotope fractionation data. According to the model proposed by Sharkey and Berry (1985), variations in ε_p are mainly determined by the carbon source taken up and the so-called leakage (L), defined as the ratio of CO_2 efflux to the total C_i uptake:

$$\varepsilon_p = a \times \varepsilon_s + L \times \varepsilon_f \qquad (7)$$

In this equation, *a* represents the fractional contribution of HCO_3^- to total C_i uptake and ε_s is the equilibrium discrimination between CO_2 and HCO_3^- (approximately $-10‰$). The fractionation of the carbon-fixing enzyme RubisCO (ε_f) is assumed to be $29‰$. As HCO_3^- is about $10‰$ enriched in ^{13}C compared with CO_2 (Zeebe and Wolf-Gladrow 2001), an increasing proportion of HCO_3^- uptake diminishes ε_p, which is defined relative to CO_2 as the carbon source. If there is no change in carbon source, ε_p decreases with decreasing leakage. Based on these considerations, carbon isotope data 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, while ε_p values higher than $20‰$ rule out HCO_3^- as the only carbon source.

In our study, ε_p values were found to be in between these extreme values, being consistent with both CO_2 and HCO_3^- uptake. Moreover, ε_p decreases with increasing pH in all species (Fig. 3). This trend is consistent with results obtained by Burkhardt et al. (1999a, 1999b, 2001), who found ε_p to increase with decreasing pH in six marine diatom species. Reasons for this trend will be discussed in the following sections. According to Eqn 7, a higher contribution of HCO_3^- uptake to net fixation will reduce ε_p. The 15% higher HCO_3^- contribution at high pH observed in *N. navis-varingica* (Fig. 1) would explain only $1.5‰$ lower ε_p values, far less than the observed $6‰$ difference between pH treatments. In the other two species, the HCO_3^- contribution did not change between the pH levels (Fig. 1). Consequently, most of the variation in ε_p observed in all investigated species has to result from changes in leakage. Taking the measured ε_P (Fig. 3) as well as the estimates of *a* obtained through the C_i flux assays, leakage was calculated according to Eqn 7 (Fig. 6). The calculated values decreased with increasing pH in all species, but between species, large differences were found. Highest values were calculated for *S. stellaris* (0.67 and 0.73 at high and low pH, respectively) and lowest values for *N. navis-varingica* (0.22 and 0.39 at high and low pH, respectively).

Because the calculated leakage is based on the assumption that the cell consists of a single compartment, an assumption obviously not matching the real structure of eukaryotic cells, it should be pointed out that the calculated leakage may only serve as an approximation of the maximal possible leakage. Any internal C_i cycling at the level of the chloroplast will decrease the leakage and subsequently ε_p (Schulz et al. 2007). Another shortcoming of the model by Sharkey and Berry (1985) is the assumption of a complete equilibrium of the carbonate system. According to Raven (1997), HCO_3^- is considered as the carbon source that enters

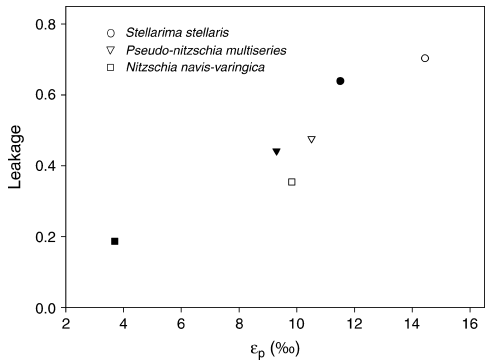

Fig. 6. Leakage of the cells as a function of ε_p, calculated according to Sharkey and Berry (1985). Closed and open symbols represent high and low pH, respectively.

the acidic thylakoid lumen (pH = 5). For such a low pH, the spontaneous rate of HCO_3^- to CO_2 conversion is sufficient to explain the observed C-fixation by RubisCO (Thoms et al. 2001). Subsequently, CA activity inside the thylakoid lumen is not necessary as previously suggested by Raven (1997). Considering that the uncatalysed conversion of HCO_3^- to CO_2 accounts for 22‰ (O'Leary et al. 1992), the CO_2 supply at the site of RubisCO in the thylakoid lumen would be isotopically lighter and hence shift ε_p to higher values. However, the latter assumes that there is no back conversion of CO_2 to HCO_3^- in the lumen, i.e. a complete non-equilibrium exists. In order to estimate the actual state of the carbonate system in the lumen, a detailed calculation is essential (Thoms et al. in preparation).

We also employed the MIMS to get estimates on leakage following the approach by Badger et al. (1994). Leakage was relatively low in all three species and diminished only in *N. navis-varingica* with increasing pH (Fig. 4). It should be noted here that these calculations are based on several assumptions, for instance, that the rate of diffusive CO_2 efflux is well represented during the first seconds of the subsequent dark phase. This approach may underestimate the real C_i efflux because of re-fixation of CO_2 by RubisCO in the dark (Badger et al. 1994, Rost et al. 2006b). Furthermore, a prerequisite for the application of this approach is the absence of eCA activity, which we ensured by the addition the eCA inhibitor DBS. Because eCA might act as a C_i-recycling mechanism, the inhibition of this enzyme may have an effect on the CO_2 efflux estimates. This underlines that new approaches are required to obtain better estimates on the important aspect of leakage.

Relationship between pH, carbon acquisition and DA production

It has been reported that higher DA production occurred at elevated pH in different strains of *P. multiseries* (Lundholm et al. 2004). To explain this effect, it has been proposed that toxin production could be induced by carbon limitation with increasing pH (Lundholm et al. 2004). In our experiments, as expected, DA was produced in the two potentially toxic species *P. multiseries* and *N. navis-varingica*, while no production was found in the non-toxic species *S. stellaris*. The content of DA increased significantly in both toxic species with increasing pH (Table 3) but was most pronounced for *P. multiseries*, where the increase in cellular DA content was more than 70-fold. This finding confirms the observation by Lundholm et al. (2004) that increasing pH induces higher DA levels. With respect to rather low values of $K_{1/2}$ for photosynthesis in *P. multiseries* and *N. navis-varingica* (Table 2), significant DIC limitation is not indicated even under high pH. Consequently, the suggested relationship between DA and carbon acquisition/C_i limitation cannot be confirmed. It is hence likely that pH triggers the DA production in another way than by altering the carbonate chemistry. As such, external pH could affect internal pH, which in turn could alter many processes that are not associated to carbon acquisition or limitation (Hansen et al. 2007). The importance of pH should therefore be considered in future monitoring programmes for harmful phytoplankton species.

Ecological implications and conclusions

In view of the ongoing acidification of the oceans (Wolf-Gladrow et al. 1999, Orr et al. 2005) as well as elevated pH during blooms (Hansen 2002), the observed differences in CCM efficiency and regulation of the investigated diatoms may play an important role for the dominance of certain diatom species (Tortell et al. 2002, Rost et al. 2003). It is a common notion that species being able to use the large HCO_3^- pool may have a competitive advantage over those that rely on CO_2 and thus may be less sensitive to variations in pH (Hansen 2002, Korb et al. 1997, Nimer et al. 1997, Tortell and Morel 2002). Consequently, elevated pH should especially favour species that prefer HCO_3^- as their carbon source. However, the pH limit for growth for the predominant HCO_3^- user *S. stellaris* was already attained at pH 8.8 (Lundholm, unpublished data) although most of the DIC was present in the form of its preferred carbon source. *S. costatum* showed a strong preference for HCO_3^- under high pH (Rost et al. 2003), but growth was already affected above pH 8.5 (Schmidt and Hansen

2001). In contrast, the CO_2 user *N. navis-varingica* grew up to pH values of 10 (Lundholm, unpublished data, Kotaki et al. 2000). Similarly, the CO_2 user *P. tricornutum* (Burkhardt et al. 2001) can maintain growth up to pH 10 (Goldman et al. 1982, Humphrey 1975). Such high pH limits for growth in CO_2 users call into question the common notion that 'HCO_3^- users' have a competitive advantage over CO_2 users. According to our data and those of previous investigations, 'HCO_3^- users' are as sensitive as CO_2 users with regard to their pH/CO_2 dependence of growth. This finding is surprising because the CO_2 availability is strongly reduced at high pH and thus CO_2 users should be *more* prone to elevated pH. The underlying mechanisms are unclear but may point to species-specific differences in leakage (Rost et al. 2006b) as well as direct effects of pH (Hansen et al. 2007). The latter could have impact on the ion balance of the cell and hence transporter functioning and energy requirements.

Bloom-forming phytoplankton species should be especially dependent on an efficient and regulated CCM as they maintain high growth rates even under bloom conditions when pH rises because of photosynthetic carbon consumption (e.g. Elzenga et al. 2000, Rost et al. 2003). The bloom-forming species *P. multiseries* obtained approximately 30% higher V_{max} of photosynthetic O_2 evolution compared with the non-bloom-forming species (Table 2). Considering that CO_2 concentrations may be as low as 5 μmol l^{-1} towards the end of a bloom, the high C_i affinity observed in bloom-forming species like *P. multiseries* directly translates to higher rates of carbon fixation. Moreover, bloom-forming diatom species tend to be more flexible in the use of different carbon sources (Fig. 1). These abilities may provide a competitive advantage, especially under changing conditions as they occur during a bloom. According to our results, the diatoms as group differ strongly in their mode of carbon acquisition and hence generalizations cannot be made.

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PUBLICATION III

THE EFFECT OF $_pCO_2$ ON THE CARBON ACQUISITION AND INTRACELLULAR ASSIMILATION IN FOUR MARINE DIATOM SPECIES[1]

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Running title: CARBON ACQUISITION IN MARINE DIATOMS

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Abstract

The effect of pCO_2 on carbon acquisition and intracellular assimilation was investigated in the three bloom-forming diatom species, *Eucampia zodiacus*, *Skeletonema costatum*, *Thalassionema nitzschioides* and the non-bloom-forming *Thalassiosira pseudonana*. *In vivo* activities of 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 pCO_2 levels of 370 and 800 μatm. To investigate whether the cells operate a C_4-like pathway, activities of ribulose-1,5-bisphosphate carboxylase (RubisCO) and phosphoenolpyruvate carboxylase (PEPC) were measured in the previous acclimations and another pCO_2 level of 50 μatm. In the bloom-forming species, extracellular CA activities strongly increased with decreasing CO_2 supply while constantly low activities were obtained for *T. pseudonana*. Half-saturation concentrations ($K_{1/2}$) for photosynthetic O_2 evolution decreased with decreasing CO_2 supply in the bloom-forming species, while for *T. pseudonana* apparent affinities were constantly high. With increasing pCO_2, maximum rates of photosynthesis (V_{max}) of the photosynthetic O_2 evolution increased in *E. zodiacus* and *S. costatum*, while V_{max} remained constant in *T. pseudonana* and *T. nitzschioides*. Independent of the pCO_2 level, PEPC activities were significantly lower than those for RubisCO, averaging generally less than 3%. All examined diatom species operate efficient and regulated CCMs, but differ strongly in the expression of individual components. The present data do not support C_4 metabolism in the investigated species.

Kex Index Words

^{13}C fractionation

C_4 photosynthesis

CO_2

carbonic anhydrase

carbon concentrating mechanism

Eucampia zodiacus

photosynthesis

Skeletonema costatum

Thalassiosira pseudonana

Thalassionema nitzschioides

Abbreviations

Bicine, N,N-Bis(2-hydroxyethyl) glycerine

CA, carbonic anhydrase

Chl *a*, chlorophyll *a*

C_i, inorganic carbon

CCM, carbon concentrating mechanism

DBS, dextran-bound sulfonamide (inhibitor for eCA)

DIC, dissolved inorganic carbon

ε_a, fractionation factor between CO_2 and HCO_3^-

ε_b, fractionation factor of HCO_3^- and CO_3^{2-}

ε_f, intrinsic fractionation factor for RubisCO

ε_p, isotope fractionation during POC formation

ε_s, equilibrium discrimination between CO_2 and HCO_3^-

eCA, extracellular CA

EDTA, ethylenediaminetetraacetic acid

iCA, intracellular CA

HEPES, (2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid

$K_{1/2}$, half-saturation concentration

MIMS, membrane-inlet mass spectrometer

PDB, PeeDee belemnite standard

PEP, phosphoenolpyruvate

PEPC, phosphoenolpyruvate carboxylase

PEPCK, phosphoenolpyruvate carboxykinase

POC, particulate organic carbon

PTFE, polytetrafluoroethylene membrane

RuBP, Ribulose bisphosphate

RubisCO, Ribulose-1,5-bisphosphate carboxylase/oxygenase

V_{max}, maximum rates (substrate-saturated)

Introduction

Diatoms are a diverse and ecologically very important group contributing up to 40% of the oceans primary production (Nelson et al 1995). Among the large diversity in this group, bloom-forming diatoms play a major role in determining the downward transport of organic carbon from surface waters to the deep ocean (Buesseler 1998). Numereous diatom species are known to bloom frequently along continental margins and in upwelling regions where the nutrient availability is high (Smetacek 1999). The occurence of high diatom abundances in nutrient-rich waters has been related to several physiological adaptations. Diatoms have evolved a nutrient storage vacuole that allows accumulating nutrients in excess of its immediate growth requirements and therewith deprives competing taxa of these essential ressources (Raven 1997). This so-called luxurary consumption allows diatoms to maintain high division rates for several generations after a pulse of nutrients.

A prerequisite for high growth ratesand the ability to form blooms is an efficient acquisition of inorganic carbon (C_i) that compensates for the catalytic inefficiency of their carbon fixing enzyme Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). This highly conserved enzyme requires CO_2 as substrate, but it has only a poor affinity for this substrate (Badger et al. 1998). Because of this imperfection, RubisCO operates far below its optimum at present-day CO_2 concentrations. To overcome the potential carbon limitation marine diatoms as well as other phytoplankton taxa operate so-called carbon concentrating mechanisms (CCMs) that enrich CO_2 at the catalytic site of RubisCO. CCMs involve active uptake of CO_2 or HCO_3^- or both. The enzyme carbonic anhydrase (CA), which accelerates the otherwise slow interconversion between HCO_3^- and CO_2, can be located both inside the cell and at the cell surface. Since the loss of the accumulated inorganic carbon (C_i) by CO_2 efflux increases energetic costs and/or decreases the efficiency of a CCM, the ability of a cell to minimize the CO_2 efflux is also an important component of the CCM (Raven & Lucas 1985, Rost et al. 2006a,b).

Studying the modes of C_i acquisition and assimilation has gained increasing interest given the need to understand the potential effect of rising atmospheric CO_2 levels on overall productivity or species composotion. The group of diatoms and especially bloom-forming representatives are of particular interest because they play a major role in determining vertical fluxes of particular material. The few existing studies on CCMs of marine diatoms suggest that this group possesses relatively efficient CCMs, especially in comparison to other phytoplankton taxa, and these processes are strongly regulated as a function of CO_2 supply (Burkhardt et al. 2001, Rost et al. 2003, Trimborn et al. 2008).

Despite this common feature, diatoms appear to display a high diversity in the way they acquire C_i. It could be shown that diatoms are able to take up both CO_2 and HCO_3^-, but species differ strongly in the extent to which both carbon sources are utilized varies strongly (Burkhardt et al. 2001, Rost et al. 2003, Trimborn et al. 2008). Regarding activites of extracellular CA (eCA), diatom species also differed strongly in these studies ranging from activities close to detection limit to some of the highest reported values (Burkhardt et al. 2001, Rost et al. 2003, Trimborn et al. 2008). As pointed out by Trimborn et al. (2008), predominant uptake of HCO_3^- or CO_2 correlated generally with high or low eCA activities, respectively. Opposing the common notion that eCA functions to supply CO_2 to the uptake systems, it was suggested that eCA allows for more efficient C_i-recycling in HCO_3^- users. Also controversially discussed in diatoms is the potential role of a C_4-like photosynthetic pathway within carbon assimilation (Reinfelder et al. 2000, 2004). This involves the formation of oxaloacetate and malate by phosphoenolpyruvate carboxylase (PEPC), which has the advantage over RubisCO of a high affinity to its carbon source HCO_3^- along with insensitivity to O_2. Until now, evidence for such a pathway has only be found in the marine diatom *Thalassiosira weissflogii* (Reinfelder et al. 2000, 2004, Morel et al. 2002, Roberts et al. 2007a,b) while there are conflicting data for *Thalassiosira pseudonana* (Granum et al. 2005, Roberts et al. 2007a,b, McGinn & Morel 2008), and bloom-forming diatom genera have yet not been tested.

The aim of this study was to improve our understanding of the modes of carbon acquisition as well as to clarify whether a C_4-like pathway may operate in four diatom species. As bloom-forming representatives we chose *Eucampia zodiacus*, *Skeletonema costatum* and *Thalassionema nitzschiodes* and as non-bloom-forming species the coastal marine diatom *Thalassiosira pseudonana* for which the genome has been recently sequenced (Armbrust et al. 2004). Photosynthetic O_2 evolution and CO_2 and HCO_3^- uptake were quantified by means of a membrane-inlet mass spectrometer (MIMS) during steady-state photosynthesis. To further characterise the CCM of each species, measurements of intracellular and extracellular CA activities were performed by monitoring ^{18}O exchange from doubly labelled $^{13}C^{18}O_2$. RubisCO and PEPC activities were measured to provide insights into the biochemical mechanisms of intracellular C assimilation.

Material and Methods

Culture and experimental conditions

Eucampia zodiacus and *Thalassionema nitzschioides* (isolated from the North Sea by Anne Schwaderer, 2004), *Skeletonema costatum* (CCMP 1332) and *Thalassiosira pseudonana* (CCMP 1335) were grown at 15°C in semi-continuous cultures in sterile filtered (0.2 μm) unbuffered seawater, enriched with nutrients, silicate, trace metals and vitamins according to f/2 medium (Guillard and Ryther 1962). Experiments were carried out using a light:dark cycle of 16:8h at an incident light intensity of 200 μmol photons m^{-2} s^{-1}. Cultures were continously sparged with air containing CO_2 partial pressures (pCO_2) of 50, 370, 800 μatm resulting in pH values of 8.9, 8.2, and 7.9, respectively. pH was measured using a pH-ion-meter (WTW, model pMX 3000/pH, Weilheim, Germany) that was calibrated (2-point calibration) on a daily basis. Daily dilutions with the corresponding acclimation media ensured that the pH level remained constant (±0.05 units) and that the cells stayed in the mid-exponential growth phase. Cultures in which the pH has shifted significantly in comparison to cell-free medium at the respective pCO_2 were excluded from further analysis.

Determination of seawater carbonate chemistry

Alkalinity samples were taken from the filtrate (Whatman GFF filter, ~0.6 μm), stored in 300-mL borosilicate flasks at 4°C and measured in triplicate by potentiometric titration with an average precision of 8 μmol kg^{-1} (Brewer et al. 1986). Total alkalinity was calculated from linear Gran Plots (Gran 1952). The carbonate system was calculated from alkalinity, pH, silicate, phosphate, temperature, and salinity using the CO2Sys program (Lewis and Wallace 1998). Equilibrium constants of Mehrbach et al. (1973) refitted by Dickson and Millero (1987) were chosen. The parameters of the carbonate system for the respective treatments are given in Table 1.

Sampling

After acclimation for at least 3 days, cells acclimated to 370 and 800 μatm were harvested by gentle filtration over a 3 μm membrane filter (Isopore, Millipore). Subsequently, the cells were washed with CO_2-free f/2 medium buffered with 50 mmol L^{-1} 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES, pH 8.0). The samples were then used for measuring inorganic carbon (C_i) fluxes and CA activities with the MIMS. Samples for determination of chlorophyll *a* (Chl *a*) concentration were taken after the measurements and stored at -80°C. Chl *a* was subsequently extracted in 10 mL acetone (overnight in darkness,

at 4°C) and determined with a Turner Designs Fluorometer (Model 10-000 R, Mt. View, Canada).

Determination of CA activity

Activity of extracellular and intracellular CA was determined by measuring the loss of ^{18}O from doubly labelled $^{13}C^{18}O_2$ to water caused by the interconversion of CO_2 and HCO_3^- (Silverman 1982). The determination of CA activity was performed with a sector field multicollector mass spectrometer (Isoprime, GV Instruments, Manchester, UK) via a gas-permeable polytetrafluoroethylene membrane (PTFE, 0.01 mm) inlet system. The reaction sequence of ^{18}O loss from intial $^{13}C^{18}O^{18}O$ (m/z = 49), via the intermediate $^{13}C^{18}O^{16}O$ (m/z = 47) to the final isotopomer $^{13}C^{16}O^{16}O$ (m/z = 45) was recorded continuously. The ^{18}O enrichment was calculated as:

$$^{18}\mathrm{O}\ \log(\text{enrichment}) = \log \frac{(^{13}\mathrm{C}^{18}\mathrm{O}_2) \times 100}{^{13}\mathrm{CO}_2} = \log \frac{(\mathrm{m/z}\ 49) \times 100}{\mathrm{m/z}\ 45 + \mathrm{m/z}\ 47 + \mathrm{m/z}\ 49} \qquad (1)$$

CA measurements were performed in 8 mL f/2 medium buffered with 50 mmol L^{-1} HEPES (pH 8.0) at 15°C. To avoid interference with light-dependent C_i uptake by the cells, all measurements were carried out in the dark. After adding $NaH^{13}C^{18}O$ to a final concentration of 1 mmol L^{-1} and chemical equilibration, the uncatalyzed ^{18}O loss was monitored for about 8 minutes prior to the addition of cells. Extracellular CA activity (eCA) was calculated from the increasing rate of ^{18}O depletion after addition of the cells (slope S_2) in comparison to the uncatalysed reaction (slope S_1) and normalized on a Chl *a* basis (Badger and Price 1989):

$$\mathrm{U} = \frac{(\mathrm{S_2} - \mathrm{S_1}) \times 100}{\mathrm{S_1} \times \mu\mathrm{g\ Chl}\ a} \qquad (2)$$

Intracellular CA activitiy was determined in the presence of 100 μmol L^{-1} dextran-bound sulfonamide (DBS), an inhibitor of eCA. The drop in the log(enrichment) was calculated by extrapolation of S_2 back to the time of cell injection (Δ as defined by Palmqvist et al. 1994). Values of Δ are expressed in arbitrary units per μg Chl *a*. Chl *a* concentrations in CA assays ranged from 0.11 to 1.16 μg mL^{-1}.

Determination of net photosynthesis, CO_2 and HCO_3^- uptake

The C_i fluxes were determined during steady-state photosynthesis with the same membrane inlet mass spectrometer as for the CA measurements. The method established by Badger et al. (1994) uses the chemical disequilibrium between CO_2 and HCO_3^- fluxes during light-dependent C_i uptake to differentiate between CO_2 and HCO_3^- fluxes across the plasmalemma. C_i flux estimates are based on simultaneous measurements of O_2 and CO_2 during consecutive light and dark intervals. During dark intervals, known amounts of C_i are added to measure rates as a function of CO_2 and HCO_3^- concentrations. Rates of O_2 consumption in the dark and O_2 evolution in the light provide a direct estimate of respiration and net C_i fixation under the assumption of a respiratory quotient of 1 and a photosynthetic quotient of 1.1 to convert O_2 fluxes into C_i fluxes. Net CO_2 uptake is calculated from the steady-state rate of CO_2 depletion at the end of the light period, corrected for the CO_2/HCO_3^- interconversion in the medium. The HCO_3^- uptake is derived by a mass balance equation, i.e. the difference between net C_i fixation and net CO_2 uptake. All measurements were performed in initially CO_2-free f/2 medium buffered with 50 mmol L^{-1} HEPES (pH 8.0) at 15°C. DBS was added to the cuvette to a final concentration of 100 μmol L^{-1} to ensure complete inhibition of any eCA activity. Light and dark intervals during the assay lasted 6 minutes. The incident photon flux density was 300 μmol photons m^{-2} s^{-1}. Further details on the method and calculations are given in Badger et al. (1994) and Rost et al. (2007). Chl *a* concentrations in the assay ranged from 0.54 to 1.58 μg mL^{-1}.

Carbon isotope fractionation

Samples for particulate organic carbon (POC) were filtered onto precombusted (500°C, 12h) GFF filters (~ 0.6 μm) and stored in precombusted (500°C, 12h) Petri dishes at -20°C. Prior to analysis, POC filters were fumed with HCl for 2h to remove all inorganic carbon. POC and related $\delta^{13}C$ values were subsequently measured in duplicate on an EA mass spectrometer (ANCA-SL 2020, Sercon Ltd, Crewe, UK), with a precision ±0.5‰, respectively. The isotopic composition is reported relative to the PeeDee belemnite standard (PDB):

$$\delta^{13}C_{Sample} = \left[\frac{\left(^{13}C/^{12}C\right)_{Sample}}{\left(^{13}C/^{12}C\right)_{PDB}} - 1 \right] \times 1000 \qquad (3)$$

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

$$\varepsilon_p = \frac{\delta^{13}C_{CO_2} - \delta^{13}C_{POC}}{1 + \frac{\delta^{13}C_{POC}}{1000}} \quad (4)$$

To determine isotopic composition of DIC ($\delta^{13}C_{DIC}$), samples were sterile-filtered (0.2 μm), fixed with $HgCl_2$ (~140 mg L^{-1} final concentration), and stored at 4°C. Measurements of $\delta^{13}C_{DIC}$ were performed with a Finnegan mass spectrometer (MAT 252) at a precision of $\delta^{13}C = \pm 0.05‰$. The isotopic composition of CO_2 ($\delta^{13}C_{CO2}$) was calculated from $\delta^{13}C_{DIC}$, making use of a mass balance relation (see Zeebe and Wolf-Gladrow 2001):

$$\delta^{13}C_{HCO_3^-} = \frac{\delta^{13}C_{DIC}\,[DIC] - (\varepsilon_a[CO_2] + \varepsilon_b[CO_3^{2-}])}{(1 + \varepsilon_a \times 10^{-3})[CO_2] + [HCO_3^-] + (1 + \varepsilon_b \times 10^{-3})[CO_3^{2-}]} \quad (5)$$

$$\delta^{13}C_{CO_2} = \delta^{13}C_{HCO_3^-}(1 + \varepsilon_a \times 10^{-3}) + \varepsilon_a \quad (6)$$

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 et al. (1995), respectively.

Determination of RubisCO and PEPC activities

The activities of RubisCO and PEPC were determined using ^{14}C-based assays modified from Descolas-Gros and Oriol 1992, MacIntyre et al. 1997, Reinfelder et al. 2000, Tortell et al. 2006). The assays measure the rate of ^{14}C incorporation into organic (acid stable) carbon products following the addition of $H^{14}CO_3^-/^{14}CO_2$ and ribulose bisphosphate (RuBP) or phosphoenolpyruvate (PEP). In the present study, we largely followed the protocol described by Tortell et al. (2006) with a few modifications.

Cells acclimated to 50, 370, and 800 μatm were concentrated by filtration over a 3 μm membrane filter (Isopore, Millipore). Subsequently, 15 mL of the concentrated cell suspension were transfered to a falcon tube and placed on ice. The samples were then concentrated by centrifugation at 4000 rpm (centrifuge Jouan, Model BR4i, Saint Herblain, France) for 10 min at 0°C. The pellet was resuspended with 2 mL ice-cold extraction/assay buffer and transferred into a 2 mL Apex vial. The buffer, modfied from MacIntyre et al. (1997) contained 50 mmol L^{-1} N,N-Bis(2-hydroxyethyl) glycerine (BICINE, pH 7.5), 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), 10 mmol L^{-1} $MgCl_2$, 1.5 mol L^{-1} glycerol, 10 mmol L^{-1} $NaHCO_3$, 5 mg L^{-1} bovine serum albumen, 0.2% Triton-X, and 5 mmol L^{-1} dithiotrietol (DTT). The samples were then homogenized in a glass grinding tube, which was placed in an ice-containing tumbler, with a rotating glass potter (EUROSTAR digital,

IKA-Werke, Staufen, Germany) at 1000 rpm for 3 intervals of 30 s. Subsequently, samples were sonicated (Branson Sonifier 450, Schwäbisch Gmünd, Germany) with a microtip at 70% duty cycle for 3 intervals of 30 s at -2°C. Crude cell extracts were then clarified by centrifugation (Centrifuge Hettich, Mikro 22R, Schnakenberg, Germany) at 14000 rpm for 30 s at 0°C, and the supernatants retained for enzyme assays.

After extraction, seven 200 μL aliquots were taken from the supernatant and dispensed into a microtip, two replicates each for blank, RubisCO and PEPC activity. Then, samples were preincubated over 15 min in the dark to ensure that any residual RuBP and PEP pools have been depleted. With the exception of the blank, 20 μL of either the RuBP stock (23 mmol L^{-1}) or the PEP stock (50 mmol L^{-1}) was added to the subsamples. Stock solutions of RuBP and PEP were both stored frozen at -20°C. After a 3-min incubation at room temperature, a 5 μCi spike of $NaH^{14}CO_3^-$ (CFA3, GE Healthcare, Freiburg, Germany) was injected into all samples to initiate ^{14}C fixation. After 30 min, reactions were terminated by the addition of 100 μL HCl (6 mol L^{-1}). To remove residual inorganic ^{14}C that had not been fixed, samples were placed in a fume hood on a shaker table and left to degas for at least 24 h. Degassed samples were then transferred into 7-mL scintillation vials and 5 mL of scintillation cocktail (Ultima Gold AB, Perkin Elmer, Boston, MA, USA) was added. Then, ^{14}C was measured by means of the scintillation counter TriCarb 2100 TR (Canberra, Australia). Radioactivity in the blanks (^{14}C added without substrates) was then subtracted from all samples.

Results

Photosynthesis and C_i fluxes

Net photosynthesis, net CO_2 uptake and HCO_3^- uptake are shown as a function of CO_2 and/or HCO_3^- concentration for *E. zodiacus*, *S. costatum*, *T. nitzschioides*, and *T. pseudonana* (Fig. 1-4) acclimated to ambient (370 μatm) and high (800 μatm) pCO_2 levels. Simulteaneous uptake of CO_2 and HCO_3^- during steady-state photosynthesis was observed in all investigated species. The corresponding kinetic parameters such as half-saturation concentrations ($K_{1/2}$) and maximum rates (V_{max}) were obtained from a Michaelis-Menten fit and are summarized in Table 2.

With the exception of *T. pseudonana*, $K_{1/2}$ values for photosynthesis decreased in all investigated species with decreasing pCO_2 in the acclimation (Fig. 1-4, Table 2). With values between 1.9 and 4.0 μmol L^{-1} CO_2, the $K_{1/2}$ (CO_2) values for photosynthesis were about one order of magnitude lower than the K_M (CO_2) values known for RubisCO in marine diatoms (~31 - 41 μmol L^{-1} CO_2, Badger et al. 1998). The V_{max} of photosynthesis remained constant in *T. nitzschioides* and *T. pseudonana* while V_{max} increased with increasing pCO_2 in *E. zodiacus* and *S. costatum* (Fig. 1-4, Table 2).

The $K_{1/2}$ and V_{max} for net CO_2 uptake remained constant in *T. nitzschioides* and *T. pseudonana* independent of the pCO_2 level while both parameters increased with increasing pCO_2 in *E. zodiacus* and *S. costatum* (Fig. 1-4, Table 2). Among the investigated species, *T. pseudonana* displayed highest V_{max} for net CO_2 uptake. $K_{1/2}$ values for HCO_3^- uptake strongly decreased in all investigated species with decreasing pCO_2 with the exception of *T. pseudonana*, for which the affinities remained unaffected by the pCO_2 level. In *E. zodiacus* and *S. costatum*, V_{max} of HCO_3^- uptake increased with increasing pCO_2 level, while V_{max} remained constant in *T. nitzschioides* and *T. pseudonana.*

Using the uptake kinetics obtained in the assay, the contribution of HCO_3^- uptake relative to carbon fixation was estimated (Fig. 5). At the ambient pCO_2 level, *E. zodiacus* and *S. costatum* obtained highest relative HCO_3^- contribution with ~80% while at elevated pCO_2 both carbon sources contributed equally to net fixation. For *T. nitzschioides* and *T. pseudonana* the contribution of HCO_3^- to net fixation was ~50% independent of the pCO_2 in the acclimation.

Extra- and intracellular CA activity

With the exception of *T. pseudonana*, for which eCA activities remained constantly low, eCA activities strongly increased with decreasing pCO_2 in the other investigated species (Fig. 6a). In comparison, highest eCA activities were exhibited by *E. zodiacus* with values of ~940 U

(μg Chl *a*)$^{-1}$ at ambient CO_2 concentrations and lowest by *T. pseudonana* with values of ~120 U (μg Chl *a*)$^{-1}$. Intracellular CA activities were largely unaffected by the pCO_2 in the acclimation (Fig. 6b). In all investigated species, iCA activities were similarly high.

Activities of PEPC and RubisCO

The activities of PEPC averaged generally less than 3% of those observed for RubisCO. Moreover, the PEPC/RubisCO ratio did not change with the pCO_2 in the acclimation (Fig. 7a-d).

Carbon isotope fractionation

With the exception of *T. nitzschioides* and *S. costatum*, carbon isotope fractionation was not affected by pCO_2 (Fig. 8). While *S. costatum* and *T. nitzschioides* obtained highest fractionation with values up to 15.6‰, for *E. zodiacus* and *T. pseudonana* ε_p values were ~9.5‰.

Discussion

In the present study, we investigated the carbon acquisition and the intracellular assimilation in three bloom-forming diatoms and *T. pseudonana* in response to changes in CO_2 supply. By means of MIMS techniques in combination with ^{14}C-based assays and analyses of ^{13}C fractionation, the CCM was characterised in each species. C_i uptake kinetics and extracellular CA activities were highly regulated in the investigated bloom-forming species while *T. pseudonana* displayed a very efficient and yet constitutively expressed CCM under the investigated pCO_2 (Table 2, Fig. 1-4).

Photosynthetic O_2 evolution. The $K_{1/2}$ (CO_2) values for photosynthesis ranged between 1.9 and 4.0 μmol L^{-1} CO_2 in the investigated species (Table 2, Fig. 1-4). Being significantly lower than K_M (CO_2) of the few investigated RubisCOs of diatoms (~31 - 41 μmol L^{-1} CO_2, Badger et al. 1998) the measured apparent high affinities suggest accumulation of CO_2 at RubisCO. Another indication for the operation of a CCM in the investigated species are the changes in affinities as a function of the acclimation conditions. This up- and down-regulation of the CCM in response to the CO_2 supply has been observed in the investigated bloom-forming diatoms, but not in *T. pseudonana* at the investigated pCO_2 levels. As for other marine diatom species, our findings are consistent with previous $K_{1/2}$ values for photosynthesis obtained by MIMS techniques (Burkhardt et al. 2001, Rost et al. 2003, Trimborn et al. 2008) or by measurements of the photosynthetic O_2 evolution rate in response to varying C_i concentrations (Burns and Beardall 1987, Colman and Rotatore 1995).

Rost et al. (2003) demonstrated for a strain of *S. costatum* (an isolate from the North Sea) that $K_{1/2}$ (CO_2) values for photosynthesis were ~250 μmol L^{-1} DIC at ambient pCO_2, and 500 μmol DIC L^{-1} at 1800 μatm $_pCO_2$, which are similar to affinities obtained in the present study. Using the same strain of *T. pseudonana*, Fielding et al. (1998) performed photosynthetic kinetic experiments by acclimating cells to a range of different DIC concentrations ranging from from 0.2 to 2.75 mmol L^{-1}. They found evidence for high CCM activity and the ability of regulation in *T. pseudonana* since $K_{1/2}$ values for photosynthetic O_2 evolution varied from 85 to 470 μmol L^{-1} DIC. This is in agreement with our data on *T. pseudonana* where C_i flux measurements revealed $K_{1/2}$ values for photosynthesis of ~120 μmol L^{-1} DIC for cells that have been acclimated to 150 μatm pCO_2 under a saturating light intensity of 200 μmol m^{-2} s^{-1} (S. Trimborn, unpublished data). Overall, the change in C_i uptake kinetics for photosynthesis to ambient and high pCO_2 levels indicate that the bloom-forming species operate strongly regulated and efficient CCMs while *T. pseudonana* displayed a very efficient and yet constitutively expressed CCM.

Carbon sources and uptake kinetics. In agreement with previous studies on carbon acquisition in marine diatoms (e.g., Burns and Beardall 1987, Colman and Rotatore 1995, Rotatore et al. 1995, Korb et al. 1997), simulteaneous uptake of CO_2 and HCO_3^- was observed in the investigated diatom species (Table 2, Fig. 1-4). In addition to the estimates of the C_i sources, HCO_3^- and CO_2 uptake kinetics were determined during steady-state photosynthesis using the equations of Badger et al. (1994). According to our results, the preference for carbon species and C_i uptake kinetics differed among the investigated diatom species.

The two bloom-forming species *E. zodiacus* and *S. costatum* were characterised by a strong preference for HCO_3^- at ambient pCO_2 while both species used CO_2 and HCO_3^- in equal quantities at high pCO_2 (Fig. 5). Korb et al. (1997) demonstrated by means of ^{14}C disequilibrium technique that *S. costatum* was able to take up HCO_3^-, but did not quantify the rate or its contribution to photosynthesis. Like in the present study, Rost et al. (2003) obtained an increasing preference for HCO_3^- with decreasing CO_2 concentrations in another strain of *S. costatum*. Such an up-regulation in HCO_3^- transport might be ascribed to both an increasing number of HCO_3^- transporters and the induction of high affinity C_i uptake systems under these conditions (Table 2). In contrast to the species above, *T. nitzschioides* and *T. pseudonana* did not alter the relative contributions of HCO_3^- or CO_2 in response to changes in CO_2 supply (Fig. 5). In particular for *T. pseudonana*, the apparent affinities of the C_i uptake systems hardly responded to changes in the CO_2 supply (Table 2). According to Elzenga et al. (2000), who applied the ^{14}C disequilibrium technique, *T. pseudonana* solely relied on HCO_3^-, which stands in contrast to our results. Despite differences in the approach taken between Elzenga et al. (2000) and the present study, a recent method comparison showed that MIMS and ^{14}C-disequilibrium technique should yield identical estimates for the HCO_3^- contribution to net carbon fixation (Rost et al. 2007). The higher HCO_3^- contribution for *T. pseudonana* obtained by Elzenga et al. (2000) may have been the result of the rather high rate constants (α_1 and α_2) as well as the low CO_2 equilibrium concentration for the pH 7.0 spike used in their fit function. Based on this and previous studies, marine diatoms appear to have a high plasticity in their preference for CO_2 or HCO_3^- as well as their ability to regulate the affinities of C_i uptake systems. These large species-specific differences even exist within the group of bloom-forming diatoms and are exceptional, especially when compared to other taxa like dinoflagellates or cyanobacteria (Rost et al. 2006a, Price et al. 2007, Ratti et al. 2007).

Carbonic anhydrase activity. The enzyme carbonic anhydrase represents an important component of the CCM as it catalyses the conversion between HCO_3^- and CO_2. In agreement with previous investigations (Sültemeyer 1998, Burkhardt et al. 2001, Rost et al.

2003, Badger 2003, Trimborn et al. 2008) we found externally located CA to be up-regulated with decreasing CO_2 supply in all tested diatom species except for *T. pseudonana* (Fig. 6a). Highest eCA activities were found in the bloom-forming species with values up to 940 U (μg Chl a)$^{-1}$ for *E. zodiacus* while *T. pseudonana* displayed lowest eCA activities of 120 U (μg Chl a)$^{-1}$. These values correspond to an enhancement of the spontaneous HCO_3^-/CO_2 interconversion by 940% and 120% per μg Chl *a*. For *T. pseudonana*, the absence of significant eCA activities have also been verified using either the ^{14}C disequilibrium technique (Elzenga et al. 2000) or the electrometric method (Nimer et al. 1997). Therefore, we conclude that eCA plays an important role in the carbon acquisition of the investigated bloom-forming species while eCA activities are negligible in *T. pseudonana*.

It has been a common notion that eCA functions to increase the CO_2 concentration in the boundary layer by converting HCO_3^- to CO_2 and herewith facilitate CO_2 uptake. (e.g. Badger & Price 1994, Sültemeyer 1998, Elzenga et al. 2000, Tortell et al. 2006). However, results from model calculations indicated that eCA activities may be insufficient to significantly enhance CO_2 supply in marine microalgae (Wolf-Gladrow & Riebesell 1997). Furthermore, high eCA activities are often induced under elevated pH, hence low CO_2 equilibrium concentrations, and correlate with predominant uptake of HCO_3^- (Burkhardt et al. 2001, Rost et al. 2003, Trimborn et al. 2008, Tortell et al. 2008). Based on these observations from laboratory and field experiments, Trimborn et al. (2008) proposed that eCA acts to convert effluxing CO_2 to HCO_3^-, which is subsequently taken up via the HCO_3^- transporter. Such a C_i recycling mechanism would be most efficient when CA-mediated conversion is localized to the periplasmic space, i.e. in close vicinity of the HCO_3^- transporter. The results of the present study, i.e. high eCA activities in concert with a strong HCO_3^- preference in *E. zodiacus* and *S. costatum* (Fig. 5, 6a), are consistent with previous findings and provide, even though the novel role of eCA yet needs to be rigourously tested, further support for such a C_i recycling mechanism to operate in a large number of diatoms.

The role of intracellular CA is also under debate and its function(s) possibly differs strongly depending on the location within the cell (Badger and Price 1994, Sültemeyer 1998, Badger 2003). This is important to bear in mind because the *in vivo* approach applied in this study (Palmqvist et al 1994) does not differentiate between the various iCA forms. Furthermore, the estimates of the iCA activities rely on the diffusive influx of doubly labelled CO_2 and thus on membrane properties, intracellular pH and CO_2 concentrations as well as cell size and shape. Consequently, Δ values have arbitrary units and a direct species comparison should be treated with caution. In the present study, all four diatom species contained iCA regardless of the growth condition (Fig. 6b). Opposed to Burkhardt et al.

(2001) who found a gradual increase in iCA activity with decreasing pCO_2 in the acclimation, results of our (Fig. 6b) and previous investigations (Palmqvist et al. 1994, Rost et al. 2003, Trimborn et al. 2008) could not support this finding. Trimborn et al. (2008) suggested that iCA may be involved in a mechanism reducing the efflux from the cell. Thereafter, species predominantly relying on HCO_3^- would have low iCA activities to prevent the HCO_3^- taken up from being converted to CO_2. In contrast, species predominantly taking up CO_2 would have rather high iCA activities to equilibrate CO_2 quickly into HCO_3^- and thus preventing it from leaking out of the cell. As shown in Fig. 6b, iCA activities were similarly high irrespective of the preferred carbon source (Fig. 5). Hence, the present data do not support the proposed CO_2 trapping mechanism by Trimborn et al. (2008). However, considering the methodological uncertainities about absolute activities and location of iCA, other technical approaches have to be applied to clarify the role of iCA in carbon acquisition.

The role of C_4-like photosynthesis in marine diatoms. Evidence for unicellular C_4-like photosynthesis came from short term ^{14}C-labelling experiments (Reinfelder et al. 2000, Morel et al. 2002) and experiments with a PEPC inhibitor with the marine diatom *T. weissflogii* (Reinfelder et al. 2004). Reinfelder et al. (2000) suggested that PEPC is the primary carboxylase in the cytoplasm that forms C_4 compounds from PEP and HCO_3^-. The C_4 compound malate/aspartate is then transported into the chloroplast and decarboxylated by phosphoenolpyruvate carboxykinase (PEPCK) in close proximity of RubisCO to support carbon fixation. Reinfelder et al. (2000) demonstrated that PEPC activity was up-regulated at low CO_2 concentrations in *T. weissflogii* and that the measured PEPC activities contributed up to 50% to carbon fixation under zinc limitation. Even though the assay applied by Reinfelder et al. (2000) does not exclude the anapleurotic role of PEPC, which is considered to be involved in the synthesis of amino-acid precursors (Descolas-Gros & Oriol 1992), the observation that ^{14}C labelled malate was so rapidly formed in *T. weissflogii* (Reinfelder et al. 2000, Morel et al. 2002, Roberts et al. 2007) indicate photosynthetic C_4 fixation rather than anaplerotic processes.

In the current study, using the same experimental ^{14}C-based assay as Reinfelder et al. (2000), the PEPC/RubisCO ratios indicate little PEPC activities relative to carbon fixation by RubisCO, being generally lower than 3% (Fig. 7). Moreover, the PEPC/RubisCO ratio did not increase with decreasing pCO_2 in any of the tested diatom species. Our low PEPC/RubisCO ratios are consistent with values obtained in laboratory experiments with *P. tricornutum* (Cassar & Laws 2007) and in field studies with diatom-dominated phytoplankton assemblages (Tortell et al. 2006). The lack of significant PEPC activity in *T. pseudonana* (Fig. 7) is in agreement with findings by Granum et al. (2005) and Roberts et al. (2007). Granum

et al. (2005) revealed same levels of PEPC expression in *T. pseudonana* cells grown at 400 and 100 μatm pCO_2 using qPCR. Roberts et al. (2007) demonstrated that *T. pseudonana* exclusively relies on C_3 photosynthesis even under low CO_2 concentrations either by performing ^{14}C short-term incubations as well as by measuring gene transcripts and protein abundances of C_4-metabolic enzymes. These findings stand in contrast to McGinn & Morel (2008) postulating the prevalence of a C_4-like pathway in *T. pseudonana* and *P. tricornutum* based on analysis of gene transcripts of PEPC and PEPCK and inhibitor studies of these enzymes. They observed a 3-fold upregulation of PEPC transcripts in *T. pseudonana* under low pCO_2 acclimation. Note that McGinn & Morel (2008) did not analyse total protein content for the enzymes involved in C_4 metabolism by means of antibodies, as transcript levels are often an unreliable proxy for the amounts of corresponding functional enzymes (Gibon et al 2004).

Isotopic composition of autotrophs reflects changes in carbon fluxes as well as carbon assimilation pathways. Opposed to the classical C_3 photosynthesis driven by RubisCO, the C_4-pathway is known to deplete the apparent fractionation. Most of this is the result of PEPC, which has a much lower intrinsic fractionation than RubisCO and uses HCO_3^- as its carbon source. If a large part of C_i would be assimilated via PEPC prior to the fixation by RubisCO, this would lead to ε_p values approaching 0‰. In our four diatoms, ε_p values ranged between 9‰ and 16‰ (Fig. 8). These ε_p values are in agreement with previous studies investigating fractionation in diatoms (Burkhardt et al. 1999, Cassar & Laws 2007). We conclude that such high ε_p values and the observed variation in response to the CO_2 supply in the investigated species can easily be explained by the operation of a classical CCM without invoking C_4 photosynthesis (e.g., Raven and Johnston 1991, Rost et al. 2002, Trimborn et al. 2008).

Overall, the results of the present study suggest that PEPC activity does not significantly contribute to photosynthesis in the investigated species even under low CO_2 supply. In agreement to previous investigations, our combined data indicate rather an anaplerotic role of PEPC rather than being predominantly involved in C_4 fixation. According to Reinfelder at al. (2000) the operation of a C_4-like photosynthetic pathway provides a mean to significantly enhance the photosynthetic capacity under low CO_2 concentrations. However, when species operating C3 and C_4 metabolism are compared, we cannot observe the suggested advantage for the latter pathway. *S. costatum* (Rost et al. 2003), which operates C_3 metabolism according to our data, photosynthesize as efficiently as *T. weissflogii* (Burkhardt et al. 2001), for which C_4 metabolism has been shown (Reinfelder et al. 2000, 2004, Morel et al. 2002). This is indicated by the similarily low $K_{1/2}$ (CO_2) for photosynthesis under low CO_2

concentrations (36 μatm pCO_2) being <1 μmol L^{-1} CO_2 (Burkhardt et al. 2001, Rost et al. 2003). Hence, even when the C_4 pathway plays a primary role in photosynthesis in some species, it appears to provide no competitive advantage over diatoms operating classical CCMs.

Ecological implications and conclusions. It has been proposed that the dominance of species during bloom situations may depend on their ability to operate an efficient and regulated CCM (Rost et al. 2003, Trimborn et al. 2008). Therefore, one may assume that bloom-forming species possess the most efficient and strongly regulated CCM that allows maintenance of high growth rates even under low CO_2 availability (Rost et al. 2003), while slow-growing species may not be able to compensate for decreasing CO_2 concentrations and thus become C_i limited in their growth. In the current study, the comparison of bloom-forming and non-bloom-forming diatoms revealed that the bloom-forming species operate strongly regulated and efficient CCMs while the non-bloom-forming *T. pseudonana* displayed a very efficient and yet constitutively expressed CCM at ambient and high pCO_2 levels (Fig. 1-6, Table 2). Taking into account that significantly lower $K_{1/2}$ values for photosynthesis in *T. pseudonana* were obtained when exposed to low pCO_2 (Fielding et al. 1998, S. Trimborn unpublished data), this species up-regulated its CCM. Furthermore, up-regulation of the CCM in response to low CO_2 supply has also been observed for non-bloom-forming species such as *P. tricornutum*, *T. weissflogii*, *Nitzschia navis-varingica*, and *Stellarima stellaris* (Burkhardt et al. 2001, Trimborn et al. 2008). Hence, the ability to operate an efficient and regulated CCM applies to bloom-forming as well as to non-bloom-forming diatoms. Considering further that all diatom species examined so far mainly thrive in coastal areas, reasons for the observed high degree in CCM regulation of the investigated diatoms might be partially due to their occurence in coastal areas that display regular and large changes in CO_2 levels (Hansen 2002, Hinga 2002) as well as highly variable light conditions (e.g. MacIntyre et al. 2000, Rost et al. 2006a, Lavaud et al. 2007). Oceanic species, on the other hand, might exhibit less regulatory CCM capacities.

In view of the ongoing acidification of the oceans (Wolf-Gladrow et al. 1999, Orr et al. 2005, IPCC report I 2007), the expected increase in aquatic pCO_2 may cause a down-regulation of the CCM capacity of diatoms (Fig. 1-4, 9, Table 2). This may be the result of increasing diffusive CO_2 uptake and/or reduced energetic costs of the CCM, which can be ascribed to a decrease in leakage owing to a smaller outward CO_2 gradient driving the efflux under elevated pCO_2 (e.g. Raven & Lucas 1985, Rost et al. 2006a, b). As a consequence, diatoms may have more energy to photosynthesize and to optimize their resource allocation. Considering the low $K_{1/2}$ (CO_2) values for photosynthesis in the present and other studies

focusing on marine diatoms (e.g. Burkhardt et al. 2001, Rost et al. 2003, Trimborn et al. 2008, this manuscript), photosynthetic carbon fixation rates are close to saturation (~95%) in most diatom species under the predicted CO_2 conditions (Fig. 9). Large diatoms such as *S. stellaris* may benefit to a larger extent from the projected increase in CO_2 (Fig. 9) because of their lower affinities. It should be noted however that the observed $K_{1/2}$ values for photosynthesis were obtained under a constant pH of 8.0. In the assays, the ratio of CO_2 to DIC therefore remains constant while in natural seawater an increase in CO_2 is associated with decreasing pH and corresponding changes the CO_2 to DIC ratio. Nevertheless, incubations in unbuffered waters have also yielded higher photosynthetic carbon fixation rates under elevated pCO_2 for instance in laboratory experiments with *S. costatum* (Burkhardt & Riebesell 1997). Field and mesocosm studies with natural phytoplankton communities demonstrated that elevated pCO_2 potentially affected primary productivity, phytoplankton growth as well as the taxonomic composition (Hein & Sand-Jensen 1997, Tortell et al. 2000, Tortell et al. 2002, Riebesell et al. 2007, Tortell et al. 2008). As such, high pCO_2 promoted the growth of larger chain-forming diatom species (Tortell et al. 2002, 2008). This demonstrates that the projected CO_2/pH-related changes in seawater carbonate chemistry are likely to induce a species shift within diatoms, which could translate into enhanced vertical fluxes of particulate organic carbon from surface waters to the deep ocean and representing a negative feedback on increased atmospheric CO_2.

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Table 1. Parameters of the seawater carbonate system were calculated from alkalinity, pH, silicate, phosphate, temperature, and salinity using the CO2Sys program (Lewis and Wallace 1998).

	pCO_2 (μatm)	CO_2 (μmol kg^{-1})	DIC (μmol kg^{-1})	TA (μEq kg^{-1})	pH (NBS)
High pCO_2 acclimation	803 ± 8	31 ± 0.3	2176 ± 21	2309 ± 21	7.9 ± 0.05
Ambient CO_2 acclimation	369 ± 3	14 ± 0.1	2059 ± 19	2317 ± 15	8.20 ± 0.05
Low pCO_2 acclimation	51 ± 0.2	1.9 ± 0.03	1567 ± 40	2297 ± 9	8.85 ± 0.05

Table 2. $K_{1/2}$ and V_{max} values for photosynthesis, net CO_2 uptake, and HCO_3^- uptake for *E. zodiacus*, *S. costatum*, *T. nitzschioides* and *T. pseudonana* acclimated to ambient and high CO_2 concentrations. Kinetic parameters were calculuated from a Michaelis-Menten fit to the combined data of three independent measurements. Values for $K_{1/2}$ and V_{max} are given in μmol L^{-1} and μmol (mg Chl *a*)$^{-1}$ h^{-1}, respectively. Error bars denote ± SD (n = 3).

	Photosynthesis			Net CO_2 uptake		HCO_3^- uptake	
pCO_2 (μatm)	$K_{1/2}$ (CO_2)	$K_{1/2}$ (DIC)	V_{max}	$K_{1/2}$ (CO_2)	V_{max} (CO_2)	$K_{1/2}$ (HCO_3^-)	V_{max} (HCO_3^-)
E. zodiacus							
370	2.9 ±0.4	322.9 ±53	413.8 ±19	2.6 ±0.5	123.0 ±7	140.0 ±40	274.0 ±19
800	3.6 ±0.5	411.2 ±63	453.5 ±16	6.6 ±1.4	235.8 ± 3	325 ±101	213.9 ±19
S. costatum							
370	2.8 ±0.4	265.4 ±53	309.1 ±14	2.8 ±0.4	64.8 ±6	112.9 ±22	235.8 ±10
800	3.1 ±0.4	442.7 ±74	370.6 ±14	6.0 ±0.9	207.5 ±3	383.3 ±94	168.3 ±12
T. nitzschioides							
370	1.9 ±0.6	222.7 ±41	341.7 ±23	2.7 ±1.0	194.5 ±4	130.3 ±15	149.2 ±6
800	2.7 ±0.6	379.0 ±78	364.2 ±23	3.6 ±1.6	200.2 ±3	294.1 ±77	164.4 ±12
T. pseudonana							
370	3.4 ±0.8	513.3 ±86	484.4 ±30	3.8 ±1.2	252.9 ±4	463.1 ±73	228.1 ±11
800	4.0 ±0.9	443.4 ±98	470.4 ±28	3.4 ±1.1	261.6 ±4	380.2 ±96	212.3 ±15

Figure legends

Fig. 1. *E. zodiacus*. Chl *a* specific rates of net photosynthesis, net CO_2 uptake and HCO_3^- uptake as a function of CO_2 and HCO_3^- concentration in the assay medium. The cultures were acclimated to (a) (b) 370 μatm, (c) (d) 800 μatm of CO_2 for at least 3 d. Curves were obtained from a Michaelis-Menten fit.

Fig. 2. *S. costatum*. Chl *a* specific rates of net photosynthesis, net CO_2 uptake and HCO_3^- uptake as a function of CO_2 and HCO_3^- concentration in the assay medium. The cultures were acclimated to (a) (b) 370 μatm, (c) (d) 800 μatm of CO_2 for at least 3 d. Curves were obtained from a Michaelis-Menten fit.

Fig. 3. *T. nitzschioides*. Chl *a* specific rates of net photosynthesis, net CO_2 uptake and HCO_3^- uptake as a function of CO_2 and HCO_3^- concentration in the assay medium. The cultures were acclimated to (a) (b) 370 μatm, (c) (d) 800 μatm of CO_2 for at least 3 d. Curves were obtained from a Michaelis-Menten fit.

Fig. 4. *T. pseudonana*. Chl *a* specific rates of net photosynthesis, net CO_2 uptake and HCO_3^- uptake as a function of CO_2 and HCO_3^- concentration in the assay medium. The cultures were acclimated to (a) (b) 370 μatm, (c) (d) 800 μatm of CO_2 for at least 3 d. Curves were obtained from a Michaelis-Menten fit.

Fig. 5. Ratios of HCO_3^- uptake: net photosynthesis of cells aclimated to 370 μatm and 800 μatm CO_2. Ratios from MIMS measurements were based on the rates obtained at C_i concentrations of about 2 mmol L^{-1}.

Fig. 6. Chl *a*-specific activities of (a) eCA and (b) iCA activities from cells acclimated to 370 μatm and 800 μatm CO_2. Values represent the means of triplicate incubations (± SD).

Fig. 7. The relative ratios of PEPC activity: RubisCO activity of cells aclimated to 50, 370, and 800 μatm CO_2. Error bars denote ± SD ($n \geq 3$).

Fig. 8. Isotope fractionation (ε_p) from cells acclimated to 370 μatm and 800 μatm CO_2. Values for ε_p have been calculated from the $^{13}C_{CO2}$ and $^{13}C_{POC}$ in the respective acclimations of each species. Error bars denote ± SD ($n = 3$).

Fig. 9. The range of relative photosynthesis saturation of different diatom species (according to Burkhardt et al. 2001, Rost et al. 2003, Trimborn et al. 2008, current study) is illustrated with respect to ambient CO_2 (370 μatm) and high CO_2 (up to 800 μatm). The lowest $K_{1/2}$ values was observed for *T. nitzschiodes* (1.9 μmol L^{-1} CO_2, this study) at ambient pCO_2 while the highest $K_{1/2}$ values of 7.4 μmol L^{-1} CO_2 was recorded for *S. stellaris* at high pCO_2 (Trimborn et al. 2008). Since all measured $K_{1/2}$ values for diatoms that have been acclimated to ambient and high pCO_2 levels of up to 800 μatm are included in the present figure (according to Burkhardt et al. 2001, Rost et al. 2003, Trimborn et al. 2008, current study), the range in half-saturation concentrations from photosynthesis shown here reflects the degree of CCM regulation of marine diatoms according to ambient and high CO_2 levels. Highest apparent affinities for CO_2 were observed at ambient CO_2. Extrapolating carefully the observed $K_{1/2}$ values to the carbonate chemistry in the ocean, it is likely that marine diatoms may perform between 80% and 95% of relative photosynthesis at the projected high CO_2 levels.

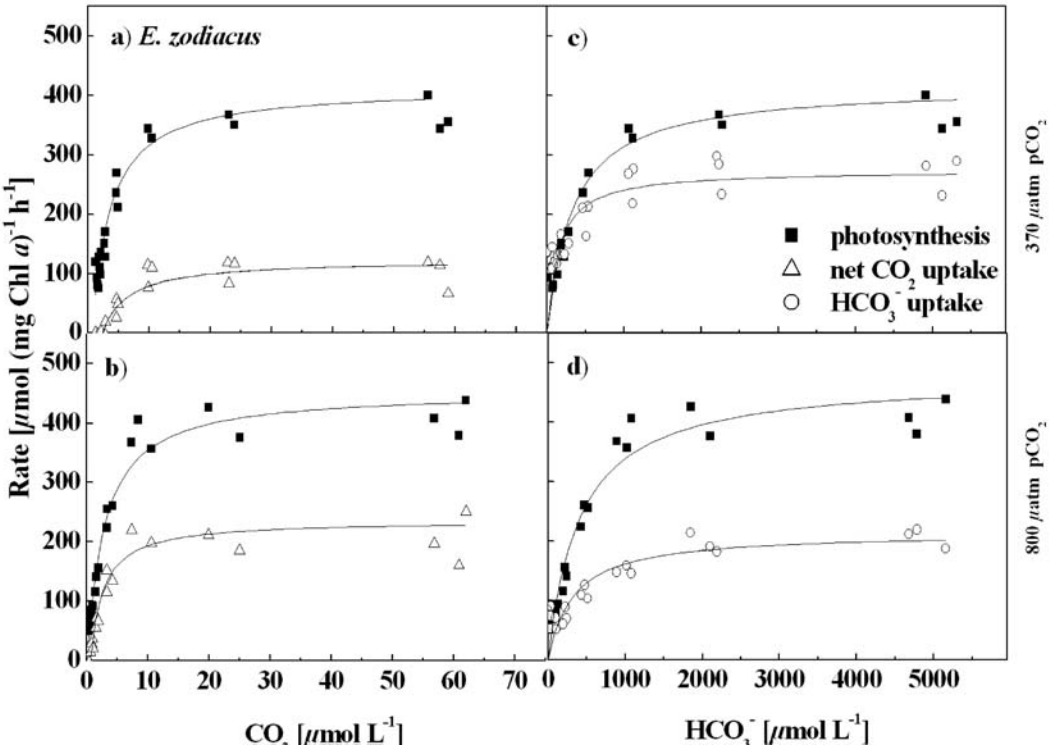
a) E. zodiacus
c)
b)
d)
photosynthesis
net CO2 uptake
HCO3- uptake
370 µatm pCO2
800 µatm pCO2
Rate [µmol (mg Chl a)-1 h-1]
CO2 [µmol L-1]
HCO3- [µmol L-1]
0
100
200
300
400
500
0
10
20
30
40
50
60
70
0
1000
2000
3000
4000
5000

Figure 1

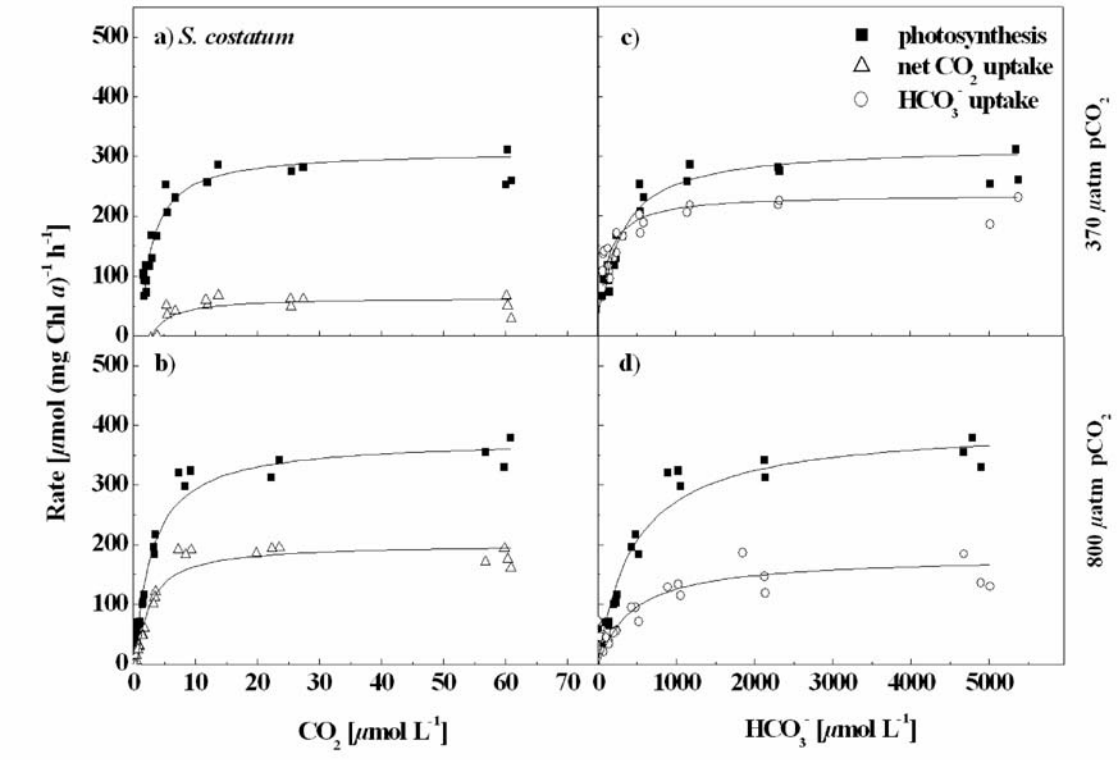
a) S. costatum
b)
c)
d)
photosynthesis
net CO2 uptake
HCO3- uptake
370 µatm pCO2
800 µatm pCO2
Rate [µmol (mg Chl a)-1 h-1]
CO2 [µmol L-1]
HCO3- [µmol L-1]
0
100
200
300
400
500
0
10
20
30
40
50
60
70
0
1000
2000
3000
4000
5000

Figure 2

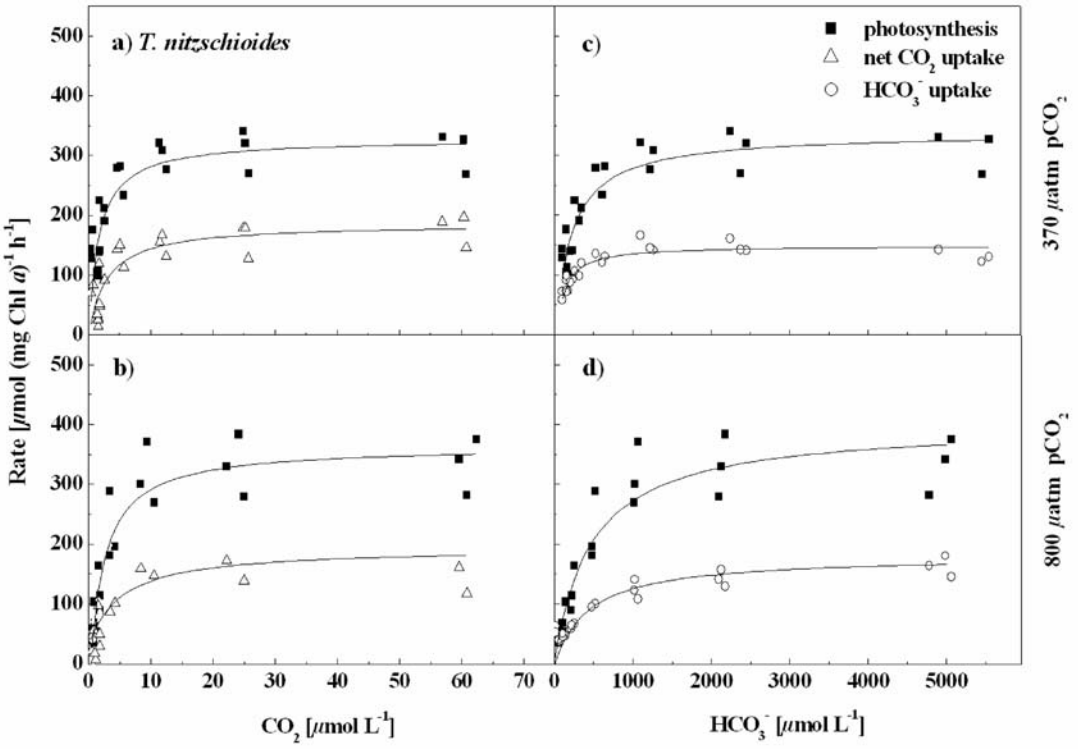
a) T. nitzschioides
c)
b)
d)
photosynthesis
net CO2 uptake
HCO3- uptake
370 µatm pCO2
800 µatm pCO2
Rate [µmol (mg Chl a)-1 h-1]
CO2 [µmol L-1]
HCO3- [µmol L-1]
0
100
200
300
400
500
0
10
20
30
40
50
60
70
0
1000
2000
3000
4000
5000

Figure 3

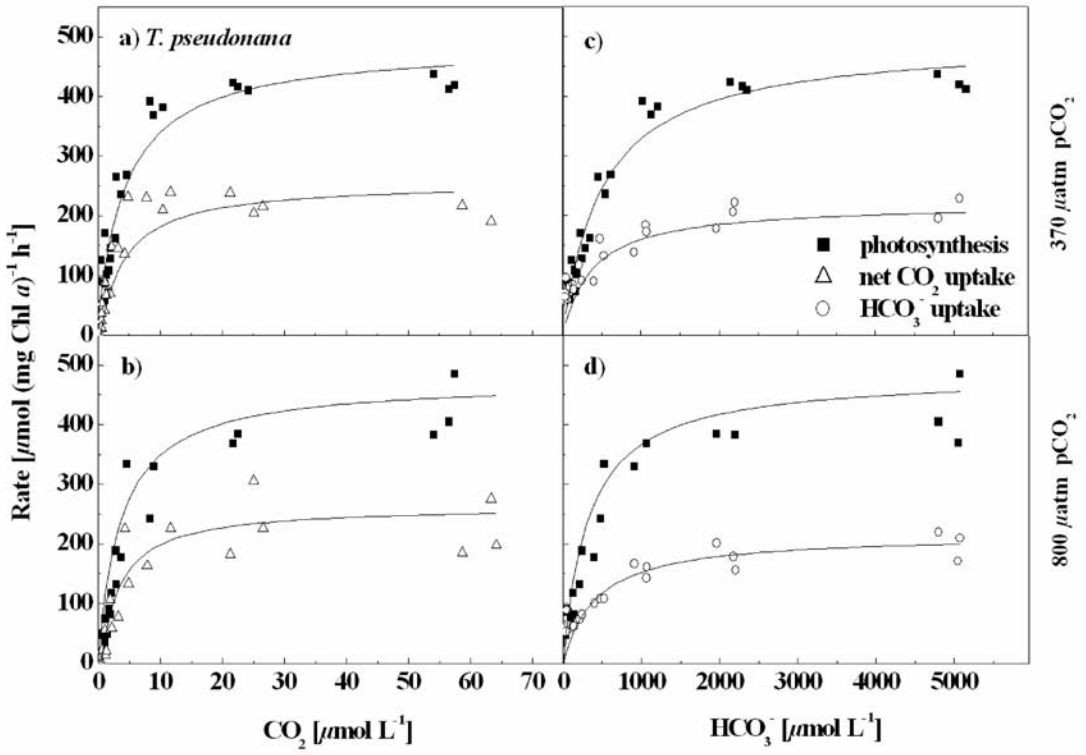
a) T. pseudonana
b)
c)
d)
photosynthesis
net CO2 uptake
HCO3- uptake
370 µatm pCO2
800 µatm pCO2
Rate [µmol (mg Chl a)-1 h-1]
CO2 [µmol L-1]
HCO3- [µmol L-1]
0
100
200
300
400
500
0
10
20
30
40
50
60
70
0
1000
2000
3000
4000
5000

Figure 4

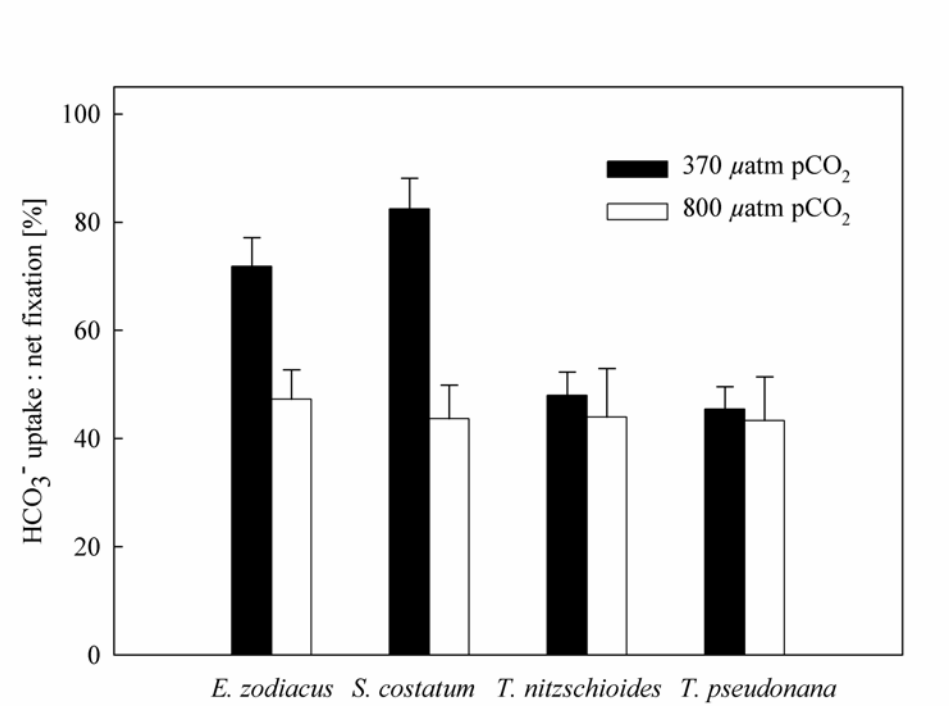
100
80
60
40
20
0
HCO_3^- uptake : net fixation [%]
370 μatm pCO_2
800 μatm pCO_2
E. zodiacus
S. costatum
T. nitzschioides
T. pseudonana

Figure 5

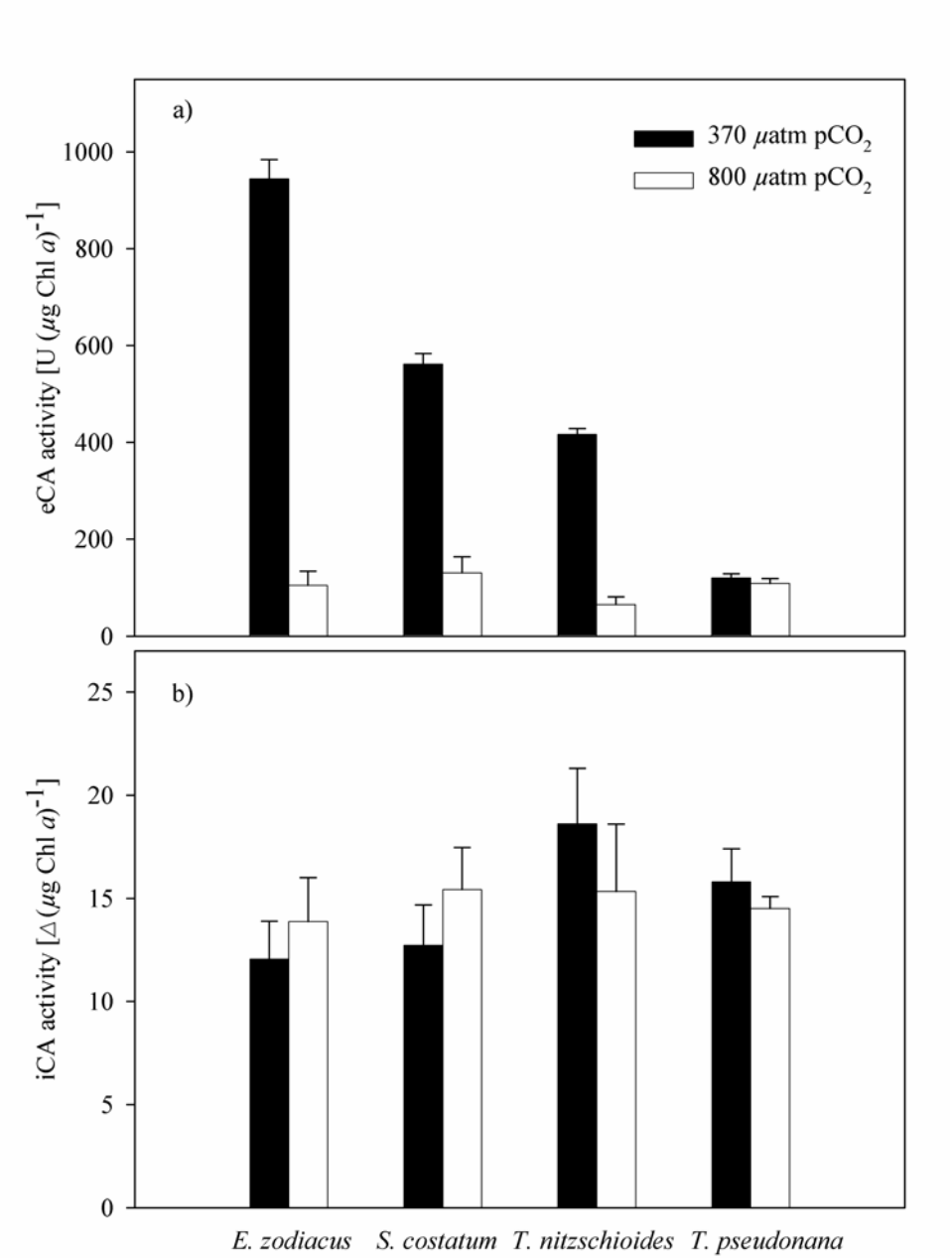
a)
370 μatm pCO2
800 μatm pCO2
eCA activity [U (μg Chl a)-1]
1000
800
600
400
200
0
b)
iCA activity [Δ (μg Chl a)-1]
25
20
15
10
5
0
E. zodiacus
S. costatum
T. nitzschioides
T. pseudonana

Figure 6

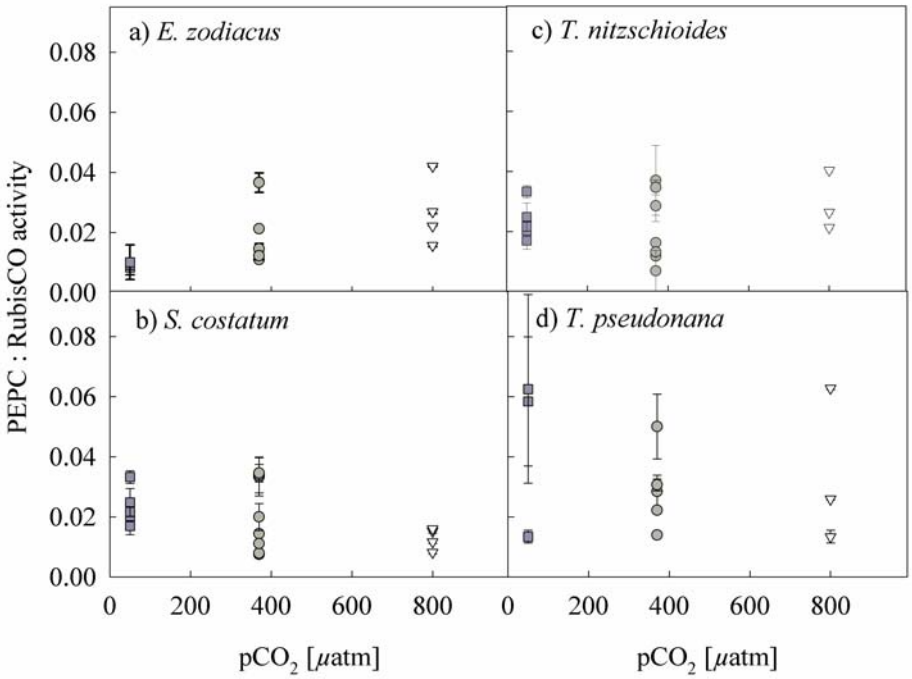
a) E. zodiacus
c) T. nitzschioides
b) S. costatum
d) T. pseudonana
PEPC : RubisCO activity
0.00
0.02
0.04
0.06
0.08
0
200
400
600
800
pCO2 [μatm]

Figure 7

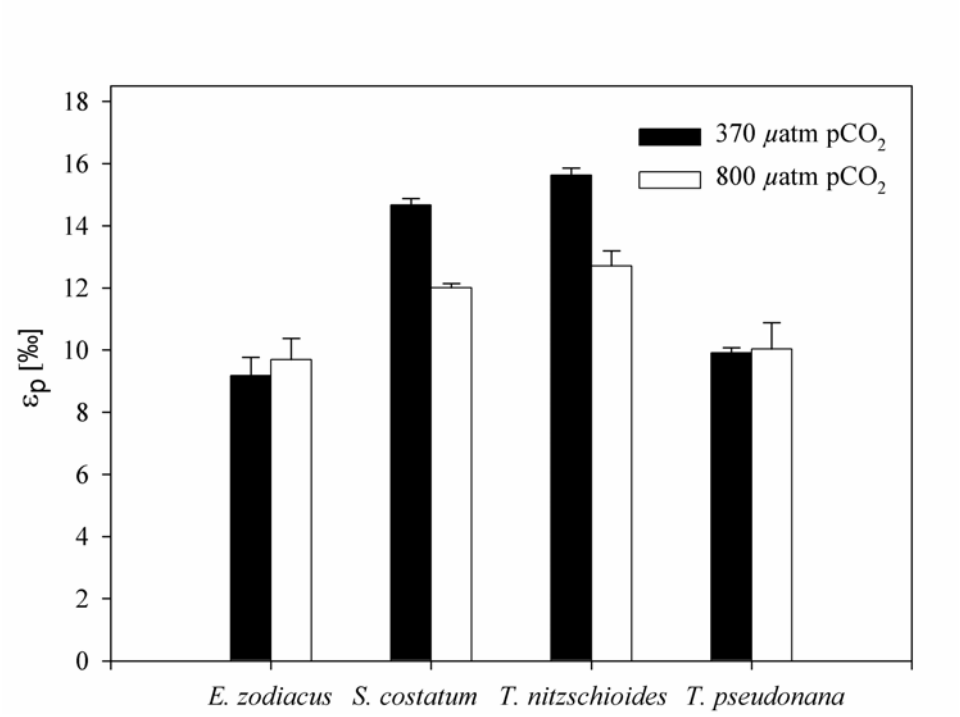
18
16
14
12
10
8
6
4
2
0
εp [‰]
370 μatm pCO2
800 μatm pCO2
E. zodiacus
S. costatum
T. nitzschioides
T. pseudonana

Figure 8

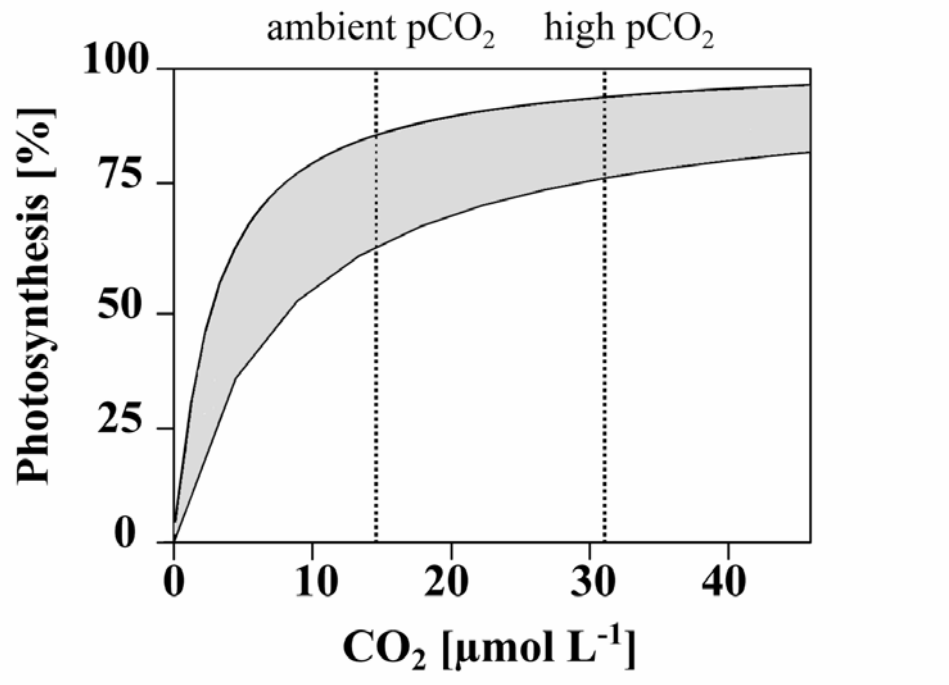
ambient pCO2
high pCO2
100
75
50
25
0
Photosynthesis [%]
0
10
20
30
40
CO2 [µmol L-1]

Figure 9

PUBLICATION IV

THE CO_2 SENSITIVITY OF SOUTHERN OCEAN PHYTOPLANKTON

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Abstract

The Southern Ocean exerts a strong impact on marine biogeochemical cycles and global air-sea CO_2 fluxes. Over the coming century, large increases in surface ocean CO_2 levels, combined with increased upper water column temperatures and stratification, are expected to diminish Southern Ocean CO_2 uptake. These effects could be significantly modulated by concomitant CO_2-dependent changes in the region's biological carbon pump. Here we show that CO_2 concentrations affect the physiology, growth and species composition of phytoplankton assemblages in the Ross Sea, Antarctica. Field results from in situ sampling and ship-board incubation experiments demonstrate that inorganic carbon uptake, steady-state productivity and diatom species composition are sensitive to CO_2 concentrations ranging from 100 to 800 ppm. Elevated CO_2 led to a measurable increase in phytoplankton productivity, promoting the growth of larger chain-forming diatoms. Our results suggest that CO_2 concentrations can influence biological carbon cycling in the Southern Ocean, thereby creating potential climate feedbacks.

1. Introduction

The Southern Ocean regulates atmospheric CO_2 concentrations over glacial-interglacial cycles [*Sigman and Boyle*, 2000] and contributes disproportionately to the oceanic sequestration of anthropogenic CO_2 [*Caldeira and Duffy*, 2000]. The availability of iron and light are believed to exert primary controls on Southern Ocean productivity and biological carbon uptake [*Boyd*, 2002]. In contrast, CO_2 has not been considered as a potentially important factor affecting phytoplankton growth and community composition in this region. Recent field studies have demonstrated CO_2 effects on phytoplankton in several oceanic regimes [*Hein and SandJensen*, 1997], [*Riebesell et al.*, 2000], [*Tortell et al.*, 2002], yet the extent to which the results apply to high latitude regions is unknown. The near freezing temperatures of Antarctic seawater significantly increase CO_2 solubility, yielding equilibrium CO_2 concentrations more than two-fold higher than those of tropical waters. Southern Ocean carbon isotope data have been interpreted as evidence of CO_2-dependent photosynthesis [*Rau et al.*, 1989], and laboratory experiments suggest that CO_2 diffusion can limit the growth rates of large Antarctic diatoms [*Riebesell et al.*, 1993]. Recent work has demonstrated, however, that at least some Southern Ocean phytoplankton possess cellular carbon concentrating mechanisms and can utilize the abundant HCO_3^- ion as an inorganic carbon (C_i) source [*Cassar et al.*, 2004]. The extent to which CO_2 concentrations can regulate C_i uptake, growth and species composition of Antarctic phytoplankton assemblages has thus far not been examined. To address these questions, we examined the CO_2-sensitivity of

phytoplankton populations in the Ross Sea, one of the most productive regions in the Southern Ocean [*Arrigo et al.*, 1998].

2. Methods

Sampling and experiments were conducted in the Ross Sea polynya during the Austral summer (December 2005 - January 2006), and Austral spring (November - December 2006). Surface water samples (5m) were collected at 35 stations to examine the physiological mechanisms of inorganic C utilization by in situ phytoplankton assemblages. Phytoplankton were concentrated by gravity filtration onto 2.0 μm pore size filters, and the fraction of cellular HCO_3^- and CO_2 uptake was measured in concentrated phytoplankton samples using isotope disequilibrium experiments at a pH of 8.5 and temperature of 2.0 °C [*Martin and Tortell*, 2006]. For a subset of samples (11 stations), chlorophyll *a*-normalized maximum C uptake rates (V_{max}) were determined by measuring 10 min ^{14}C uptake over a range of external C concentrations, and fitting data to a Michaelis-Menten hyperbolic equation. For these experiments, phytoplankton were concentrated in a C-free buffer (pH 8.0 seawater buffer sparged with CO_2-free air), and ^{14}C uptake rates measured across a range of test DIC concentrations. The maximum uptake rates (V_{max}) derived from this analysis provide an estimate of the total physiological capacity of phytoplankton for inorganic C uptake under substrate saturating conditions, and are not necessarily related to steady-state C fixation rates.

In addition to our *in situ* sampling, we also conducted CO_2 manipulation experiments with phytoplankton collected at 3 different Ross Sea locations. For these incubations, we used a semi-continuous batch-culture technique [*Tortell et al.*, 2002], where sample bottles were periodically diluted with 0.2 μm filtered, seawater to prolong exponential phytoplankton growth. For all experiments, triplicate incubations bottles were bubbled with commercially prepared air standards containing 100, 380, or 800 ppm CO_2, and amended with 1 nM Fe (as $FeCl_3$) to promote phytoplankton growth. No additional macronutrients were added to the incubation bottles. Of the three experiments, two were conducted in a deck-board flowing seawater tank with temperatures close to in situ values (0 ± 1 °C), and light intensity reduced to ~30% of surface irradiance levels with two layers of neutral density screening. Due to very cold air temperatures at the beginning of the spring cruise (November 2006), one incubation experiment was run in a temperature controlled growth chamber, (0 °C) with a constant blue light irradiance of 165 μmol quanta m^{-2} s^{-1}. Incubation experiments lasted between 10 and 18 days depending upon logistical constraints.

For all incubation experiments, steady-state phytoplankton growth rates (d^{-1}) were determined from linear regressions of the natural logarithm of chlorophyll *a* concentrations

against time. Chlorophyll *a* concentrations were measured following standard JGOFS procedures (http://usjgofs.whoi.edu/protocols_rpt_19.html). At the end of each incubation experiment, samples were removed for the determination of net primary productivity using 24h ^{14}C incubations following standard JGOFS protocols. Additional isotope disequilibrium experiments were also conducted (as described above) on the final day of incubations to examine CO_2 effects on total inorganic carbon uptake rates and the relative utilization of HCO_3^-. Phytoplankton taxonomic composition was analyzed by microscopic examination of gluteraldehyde-preserved samples collected periodically during the incubations, using transmitted light microscopy to identify individual species and epifluoresence microscopy for quantitative cell counts.

3. Results and Discussion

Spring phytoplankton assemblages in the Ross Sea polynya were dominated by monospecific blooms of the prymnesiophyte alga *Phaeocystis antarctica*, while summer assemblages contained a more diverse mixture of *P. antarctica* and various diatom species. Carbon uptake experiments conducted with both spring and summer assemblages demonstrated that all phytoplankton had a high capacity for direct HCO_3^- transport. On average, HCO_3^- transport accounted for 83% (± 7) of total C uptake by phytoplankton, and there were no statistically significant differences between diatom and *Phaeocystis* dominated assemblages. Our results thus indicate the widespread occurrence of cellular carbon concentrating mechanisms in Ross Sea phytoplankton.

Over the range of ambient PCO_2 encountered during our surveys (120 - 410 ppm), we found a strong inverse relationship between maximum (i.e. substrate-saturated) C uptake rates (V_{max}) and surface water PCO_2 ($r^2 = 0.68$, $p = 0.002$), suggesting that phytoplankton upregulate carbon concentrating mechanisms in response to decreased surface water CO_2 concentrations. The apparent CO_2-dependent regulation of V_{max} cannot simply be explained by the co-variation between PCO_2 and Fe or light availability across our survey stations. At the stations we sampled for V_{max} determinations, mixed layer depths (calculated as the minimum depth where density exceeded surface values by 0.02 kg m^{-3}) averaged 85 ±15 m and were not correlated to surface water PCO_2 ($r^2 = 0.05$, $p = 0.52$, $n = 11$). Similarly, surface water dissolved Fe concentrations (mean 0.07 ±0.02 nmol L^{-1}) were not correlated to PCO_2 ($r^2 = 0.01$, $p = 0.38$, $n = 45$). Low Fe concentrations were likely limiting to phytoplankton across much of our survey area as indicated by low photosynthetic efficiency (F_v/F_m values of 0.05 to 0.35), and ship-board bioassay experiments. Overall, our survey

results indicate that CO_2 availability modulates phytoplankton C uptake under conditions of significant vertical mixing and Fe stress in the Ross Sea.

Our incubation experiments provided further compelling evidence for a specific CO_2 effect on C uptake by Ross Sea phytoplankton. For three independent experiments, short-term maximum C uptake capacity increased significantly under low CO_2 conditions, by a factor of ~2-fold relative to the highest CO_2 treatment (Fig. 1). Since all incubation bottles experienced a similar light, Fe and macronutrient regime, we can attribute the observed treatment effects specifically to the experimental CO_2 manipulations. Given the resource costs associated with carbon concentrating mechanisms [*Raven and Johnston*, 1991], this CO_2-dependent regulation of cellular C transport has important implications for the growth and net productivity of Ross Sea phytoplankton. To address this, we measured ^{14}C-based net primary productivity (24h) and chlorophyll *a*-based growth rates in sub-samples from our CO_2 incubation experiments. For the *Phaeocystis*-dominated springtime phytoplankton assemblages, there was a statistically significant increase in ^{14}C fixation between 100 and 380 ppm CO_2 (t-test, $p < 0.05$), but no further effects observed at 800 ppm CO_2 (Fig. 2a). Steady-state growth rates for these assemblages showed a parallel increase from 100 to 380 ppm CO_2, although this increase was not statistically significant due to variability among replicates. For the diatom-dominated summer phytoplankton assemblages, net ^{14}C fixation increased monotonically ($p < 0.05$) with increasing PCO_2 (Fig. 2b), and a regression of net ^{14}C productivity vs. PCO_2 was statistically significant at the 0.05 level. The response of chlorophyll *a*-specific diatom growth rates was generally consistent with that observed in ^{14}C, although only the increase in growth rates between 380 and 800 ppm CO_2 was statistically significant. We interpret the CO_2-dependent increase in net C fixation and growth rates as a result of lowered energetic costs of C assimilation under high CO_2 conditions where cells down-regulate the carbon concentrating mechanism (Fig. 1).

The magnitude of the CO_2-dependent growth and productivity effects we observed for Ross Sea phytoplankton (~10 - 20%) is consistent with results reported in other oceanic regions [*Hein and SandJensen*, 1997], yet significantly smaller than that observed in Fe bioassay experiments [*Coale et al.*, 2003]. Our measurements may, however, considerably underestimate the true response of net C fixation to increasing CO_2 levels as they do not account for the production of dissolved organic carbon (DOC). Previous studies have shown that DOC production can be as high as 20% of net C fixation in the Ross Sea [*Hansell and Carlson*, 1998], and recent work suggests that elevated CO_2 can increase dissolved organic carbon release by phytoplankton [*Engel et al.*, 2004]. This effect, if present

in the Ross Sea assemblages, would act to increase the magnitude of CO_2-stimulated carbon fixation.

Comparison of the CO_2 responses of spring and summer Ross Sea phytoplankton assemblages suggests some differences between diatom and *Phaeocystis*-dominated communities. Whereas all phytoplankton assemblages showed evidence of decreased net C fixation under low PCO_2 conditions, only the diatom-dominated summer assemblages showed a clear increase in both growth rates and net C fixation under the highest PCO_2 treatment (Fig. 2b). Beyond these broad taxonomic differences, microscopic examination of samples from the incubation experiments also revealed significant CO_2-dependent shifts in diatom species composition. In the summer experiments, where diatoms dominated the phytoplankton community over much of the Ross Sea, the relative abundance of the small pennate diatom *Pseudo-nitzschia subcurvata* decreased dramatically in the high CO_2 treatment, being replaced primarily by the larger chain-forming centric diatom *Chaetoceros* spp. (subgenus *Hyalochaetae*) (Fig. 3a-c). A similar CO_2-dependent increase in *Chaetoceros* abundance was also observed in the spring experiments despite the overwhelming dominance (> 90%) of *Phaeocystis* in the phytoplankton community (Fig. 3d). This CO_2-dependent species shift may be explained by the effects of PCO_2 on the expression of carbon concentrating mechanisms by the phytoplankton assemblages. Larger chain-forming *Chaetoceros* species may be at a competitive disadvantage for C uptake under low CO_2 conditions which induce an upregulation of cellular C transport (Fig. 1), and favor small cells such as *Pseudo-nitzschia* with high surface area to volume ratios.

4. Implications

The CO_2-dependent regulation of phytoplankton physiology, productivity and species assemblage composition has important biogeochemical implications. While iron supply exerts proximate control on primary productivity over large parts of the Southern Ocean [*Boyd et al.*, 2000], other variables such as light and silicic acid concentrations interact with iron to determine the relative growth rates and ultimate species composition of phytoplankton assemblages over the annual cycle [*Boyd*, 2002]. Our results show that CO_2 can also have a significant effect in controlling phytoplankton processes in the Southern Ocean. In regions subject to natural iron fertilization (through upwelling of deep waters [*Coale et al.*, 2005], aeolian input [*Cassar et al.*, 2007], island effects [*Blain et al.*, 2007], or melting sea ice [*Sedwick and DiTullio*, 1997]), increased CO_2 levels may promote an observable increase in phytoplankton productivity, specifically stimulating diatom-dominated assemblages and promoting a shift towards larger chain-forming species (Fig. 3).

Such diatom species, and in particular the chain forming *Chaetoceros* spp., are prolific bloom formers with a very high capacity for organic carbon export to the sediments[*Stickley et al.*, 2005]. Potential CO_2-dependent productivity increases and algal species shifts could thus act to increase the efficiency of the biological pump, enhancing Southern Ocean CO_2 uptake and contributing to a negative feedback on increased atmospheric CO_2. This feedback could be further amplified by predicted increases in surface water stratification [*Sarmiento et al.*, 1998] and Fe supply resulting from the melting of sea ice and glaciers [*Sedwick and DiTullio*, 1997] over the coming century.

Our results also bear relevance to the interpretation of paleoceanographic records of Southern Ocean biogeochemistry. To the extent that we can extrapolate our bottle incubation experiments to the open ocean, we suggest that the low CO_2 concentrations of the glacial Southern Ocean may have restricted the growth of larger, chain-forming diatoms despite higher iron inputs, instead favoring smaller, more weakly silicified taxa that are seldom preserved in the sediments. This effect could lead to the apparent decrease in total opal fluxes that has been reported for some glacial Antarctic sediments [*Mortlock et al.*, 1991], [*De La Rocha et al.*, 1998]. Many authors have suggested that changes in Southern Ocean phytoplankton productivity could explain part of the observed glacial atmospheric CO_2 drawdown[*Sigman and Boyle*, 2000]. Our work is the first, however, to provide direct evidence that CO_2 concentrations can, in turn, affect phytoplankton physiology and community structure in this region. Future field and modeling studies should consider CO_2 variability when attempting to understand the potential response of Southern Ocean phytoplankton to past and future global change.

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Figure legends

Figure 1. Effects of PCO_2 manipulations on carbon uptake by Ross Sea phytoplankton assemblages Panel (a) shows results of an experiment conducted in January 2006. Panels (b) and (c) show the results of two experiments conducted in November - December, 2006. ○ 100 ppm CO_2; ● 380 ppm CO_2; Δ 800 ppm CO_2. The amount of curvature in the time-course reflects the fraction of HCO_3^- utilization, while the final slope is proportional to total C uptake rates. The relative fraction of HCO_3^- utilization is 0.87, 0.9, and 0.95 for the data in panels a, b, and c, respectively, with no statistically significant differences among the CO_2 treatments.

Figure 2. Effects of PCO_2 manipulations on phytoplankton growth rates and net primary productivity. Panel (a) presents data from an incubation conducted during the spring growth season (November - December, 2006); Panel (b) presents data from the summer (January 2006). ● net primary productivity; Δ steady-state growth rates. Error bars represent standard errors of mean values. ^{14}C uptake was measured for 24 hours on the final day of incubation experiments, while phytoplankton growth rates (rate of chlorophyll *a* increase with time) were measured continually throughout the experiments.

Figure 3. Effects of CO_2 manipulations on the species composition of Ross Sea phytoplankton assemblages. Panels a-c show epifluorescence microscope photographs of summer phytoplankton assemblages cultured with (a) 100 ppm CO_2, (b) 380 ppm CO_2, or (c) 800 ppm CO_2. Panel (d) shows the relative abundance of *Chaetoceros* in all three incubations (including spring and summer). Percent abundances are relative to total diatom counts.

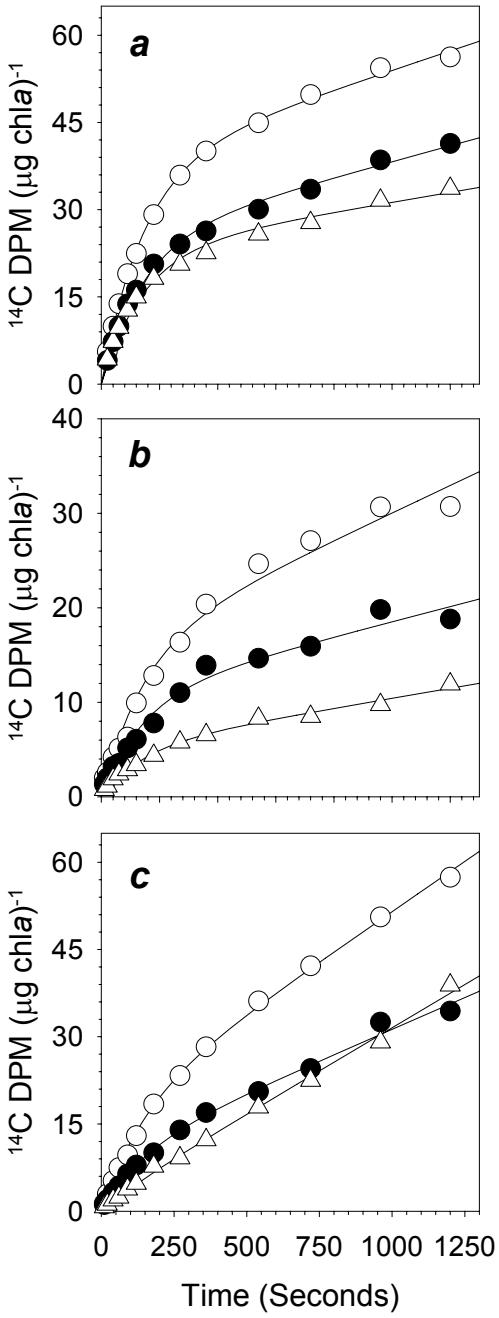

a
b
c
^{14}C DPM (µg chla)$^{-1}$
0
15
30
45
60
10
20
40
0
250
500
750
1000
1250
Time (Seconds)

Figure 1

Figure 2

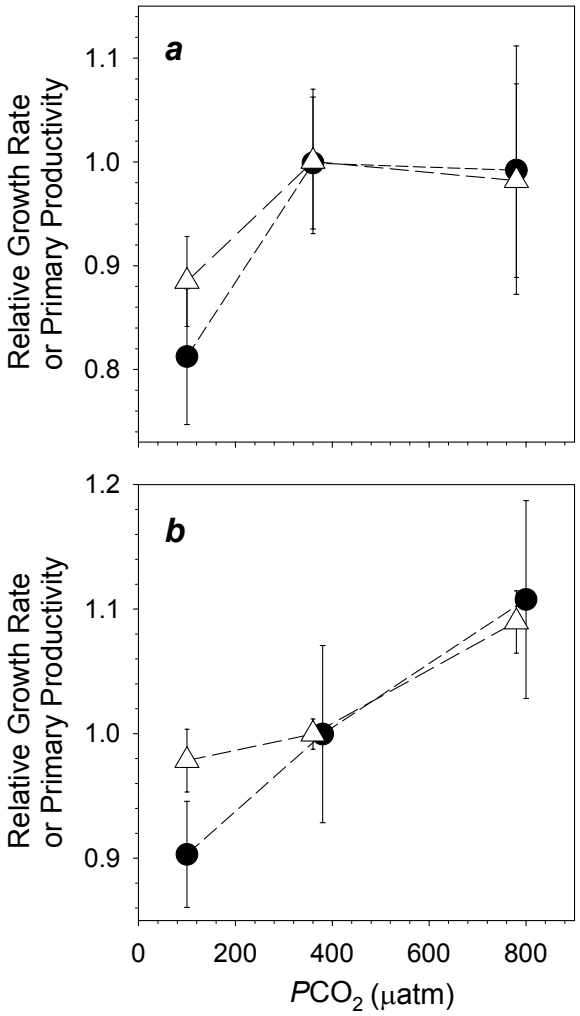
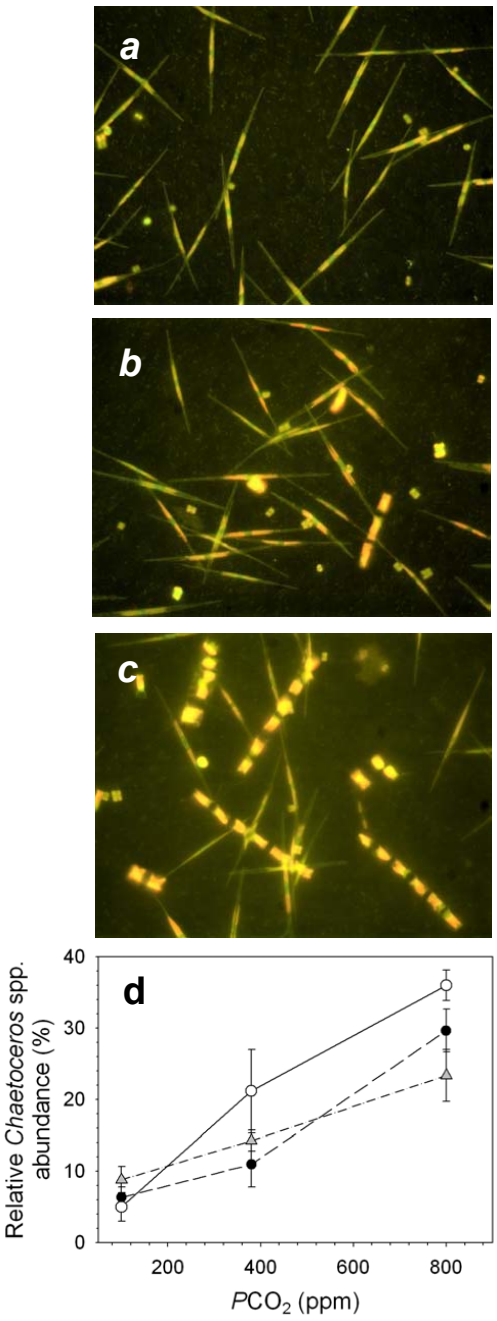

a
b
Relative Growth Rate
or Primary Productivity
1.1
1.0
0.9
0.8
1.2
0
200
400
600
800
PCO2 (μatm)

Figure 3


a
b
c
d
Relative Chaetoceros spp. abundance (%)
0
10
20
30
40
200
400
600
800
PCO2 (ppm)

5 General Discussion

4 GENERAL DISCUSSION

The publications presented in this thesis investigated the C_i acquisition of marine diatoms as well as a specific aspect of the carbon acquisition of coccolithophores. Observations from laboratory and field research delivered new insights into the efficiency and regulation of the CCM in diatoms in response to varying seawater carbonate chemistry. In the following, major issues of this thesis will be discussed from two different points of view. In the first section, the role of components of CCMs in phytoplankton ecology will be assessed. The second section concentrates on observations from the field and the laboratory. From these observations possible impacts of ocean acidification on the ecology of marine diatoms are derived. Perspectives for future research that emerge from this thesis are given at the end.

5.1 COMPONENTS OF CARBON ACQUISITION MECHANISMS AND THEIR ECOLOGICAL RELEVANCE

The role of C_i acquisition has received increasing attention in phytoplankton ecology and physiology with respect to ocean acidification as well as variations in pH during blooms. Despite the relatively high concentrations of DIC in the ocean, phytoplankton cells have to circumvent carbon limitation due to the low CO_2 supply in the present ocean and the catalytic inefficiency of RubisCO. Especially towards the end of bloom periods phytoplankton cells cause a drawdown of CO_2 due to their high photosynthetic activity. Intense photosynthetic activity can result in CO_2 values as low as 150 μatm (e.g. Codispoti et al. 1982, Bates et al. 1998). To overcome the potential carbon limitation marine microalgae operate CCMs that enrich CO_2 at the catalytic site of RubisCO and thus enhance their photosynthetic productivity. Differences in the CCM efficiency are likely to affect the competitive fitness of phytoplankton species. In this thesis, the role of different components of the CCM in marine diatoms and coccolithophores has been examined with respect to their ecological relevance.

It has been proposed that bloom-forming species should operate an efficient and regulated CCM that allows maintenance of high growth rates even under low CO_2 availability (Rost et al. 2003), while slow-growing species may not be able to compensate for decreasing CO_2 concentrations and thus become C_i limited in their growth. Apparent $K_{1/2}$ (CO_2) values for photosynthesis in the investigated diatoms were generally significantly lower than K_M (CO_2) of RubisCO in diatoms (Badger et al. 1998), clearly indicating the

operation of a highly efficient CCM (Burns & Beardall 1987, Colman & Rotatore 1995, Mitchell & Beardall 1996, Tortell et al. 1997, Burkhardt et al. 2001, Rost et al. 2003, Publication II, Publication III). By measuring the $K_{1/2}$ (CO_2) values for photosynthesis in cells that have been acclimated to varying seawater carbonate chemistry, it is further indicated whether their CCMs were down- or up-regulated under the respective experimental conditions. When exposed to low CO_2 supply, $K_{1/2}$ values for photosynthetic O_2 evolution generally decreased in the investigated diatom species (Fielding et al. 1998, Burkhardt et al. 2001, Rost et al. 2003, Publication II, Publication III) demonstrating CO_2-dependent regulation of CCMs in diatoms. The comparison of bloom-forming and non-bloom-forming diatoms revealed that both operate highly efficient and regulated CCMs (Publication II, Publication III). Considering further that the bloom-forming coccolithophore *E. huxleyi* operates a rather inefficient CCM, but yet regulated CCM in response to changes in CO_2 (Rost et al. 2003), it can be concluded that an efficient CCM is not a prerequisite for bloom-forming species. Taking into account that all diatom species examined so far mainly thrive in coastal areas, reasons for the observed high degree in CCM regulation of diatoms may be partially due to their occurrence in coastal areas that display regular changes in CO_2 (Hansen, 2002, Hinga et al. 2002) and highly variable light conditions (e.g. MacIntyre et al. 2000, Rost et al. 2006a, Lavaud et al. 2007). Consequently, oceanic species might exhibit less regulatory CCM capacities in response to CO_2. This hypothesis still needs to be tested.

The question arises whether the observed high CCM efficiency and the high degree of its regulation in marine diatoms is due to certain components of the CCM that allow them to cope with changes in the CO_2 supply. Taking into account the low CO_2 concentration typically encountered in the ocean (10-20 μmol L^{-1} CO_2) and the low K_M (CO_2) of RubisCO (Badger et al. 1998), it is evident that purely diffusive CO_2 uptake is not sufficient to explain the observed $K_{1/2}$ (CO_2) for photosynthesis in the investigated diatom species (1 - 7 μmol L^{-1}, Burns & Beardall 1987, Colman & Rotatore 1995, Mitchell & Beardall 1996, Tortell et al. 1997, Burkhardt et al. 2001, Rost et al. 2003, Publication II, Publication III). Simultaneous active uptake of CO_2 and HCO_3^- has been demonstrated in diatoms independent of changes in seawater carbonate chemistry (e.g., Burns & Beardall 1987, Rotatore et al. 1995, Korb et al. 1997, Burkhardt et al. 2001, Tortell and Morel 2002, Rost et al. 2003, Publication II, III and IV).

Despite these similarities, Publication II exemplifies that marine diatoms differ in terms of the preferred carbon source and the degree of its regulation. This stresses the importance of examining species-specific effects, especially as so-called 'model organisms'

are often considered as representatives of the whole taxon. *Stellarima stellaris* showed a strong preference for HCO_3^- ('HCO_3^- user') irrespective of the CO_2 supply, with unchanged carbon uptake kinetics. In contrast, *Nitzschia navis-varingica* is characterised by a strong preference for CO_2 ('CO_2 user') even though the contribution of HCO_3^- uptake relative to net fixation increases with decreasing CO_2 concentration, which could be ascribed to an increasing number of HCO_3^- transporters. *Pseudo-nitzschia multiseries* used CO_2 and HCO_3^- in equal quantities independent of the CO_2 concentration. *P. multiseries* compensated low CO_2 concentration during acclimation by an increased substrate affinity of the CO_2 uptake system. The increase in substrate affinity could either be due to posttranslational modifications (Sültemeyer et al. 1998) or to an increasing expression of a high affinity uptake system (e.g. Shibata et al. 2002). However, the underlying mechanisms for affinity changes have not yet been investigated to this level of detail in marine diatoms. Comparing bloom-forming and non-bloom-forming diatoms showed that diatoms generally have a high plasticity in terms of the preferred carbon source and the degree of regulation in C_i uptake enables this group to adjust the rates of C_i uptake to the actual C_i availability.

In addition to the direct uptake of HCO_3^-, extracellular carbonic anhydrase (eCA) represents an important component of the CCM as it catalyses the equilibration between HCO_3^- and CO_2. It is involved in *indirect* HCO_3^- utilisation by converting HCO_3^- to CO_2, which could then be actively transported through the plasma membrane and subsequently used for photosynthesis (*see* Badger et al. 1987). Activities of eCA were found to increase with decreasing CO_2 supply both in laboratory culture experiments (Burkhardt et al. 2001, Rost et al. 2003) and in field experiments (Berman-Frank et al. 1994, Tortell et al. 2006). The *indirect* HCO_3^- utilisation might represent an additional source of CO_2 especially for large cells since large phytoplankton are more prone to CO_2 shortage in their diffusive boundary layer under low CO_2 supply (Wolf-Gladrow and Riebesell 1997). For decades, it has been a common notion the eCA supplies CO_2 from the large HCO_3^- pool to the CO_2 uptake systems. Such a function of eCA would, however, not apply at high pH since the equilibrium is strongly on the side of HCO_3^-.

Publication II brings forward significant experimental evidence against the *indirect* HCO_3^- utilisation. In agreement with previous laboratory and field experiments (Burkhardt et al. 2001, Rost et al. 2003, Tortell et al. submitted), it was found that correlations exist between high eCA activity and predominant HCO_3^- uptake or the lack of eCA in cells predominantly taking up CO_2 (Publication II). To explain these puzzling observations, a novel hypothesis for the role of eCA was presented. According to this, the presence of eCA serves to recycle CO_2 by converting the CO_2 leaking out of the cell to HCO_3^- that is

subsequently taken up by HCO_3^- transporters. Such a mechanism would be most efficient when the CA-mediated conversion is localised in the periplasmic space, i.e. in close vicinity of the HCO_3^- transporter. It can be hypothesised that the HCO_3^- transport process is linked to the eCA activity. Support for this idea comes from red blood cells where a plasma membrane HCO_3^-/Cl^- transporter physically binds a CAII protein (Sterling et al. 2001). For 'CO_2 users', the absence of eCA may cause the CO_2 leaking out of the cell to be prevented from fast conversion to HCO_3^- and a disequilibrium at the cell surface persists. The proposed C_i recycling mechanism is further supported by data obtained from four more diatom species (Publication III). The corroboration of the potential role of eCA as a C_i recycling mechanism and the elaboration of its relevance in carbon acquisition by means of molecular biological approaches and inhibitor studies will represent major advances in this field.

Owing to their ability to access the large HCO_3^- pool, it is widely accepted that species characterised as predominant HCO_3^- users may be less sensitive to variations in pH and thus may have a competitive advantage over those that rely mainly on CO_2 (Nimer et al. 1997, Korb et al. 1997, Hansen 2002, Tortell & Morel 2002). Consequently, elevated pH should especially favour species that prefer HCO_3^- as their carbon source. However, the pH limits for growth of 'HCO_3^- users' are much smaller than for CO_2 users (Publication II). For diatom species being characterised as 'HCO_3^- users' like *S. stellaris* (Publication II), growth was already affected above pH 8.8 (unpublished data, N. Lundholm). In contrast, 'CO_2 users' such as *N. navis-varingica* and *Phaeodactylum tricornutum* are able to grow until pH values of up to 10 (unpublished data, N. Lundholm, Humphrey 1975, Goldman et al. 1982, Kotaki et al. 2000). Hence, it can be concluded that 'HCO_3^- users' are not less sensitive than 'CO_2 users' with regard to their pH/CO_2-dependence of growth (Publication II). The low pH tolerance of 'HCO_3^- users' is surprising. This might be due to direct effects of pH (*see* also Hansen et al. 2007) and differences in leakage of CO_2. The latter aspect will be discussed in the following.

The capability of species to minimise the loss of CO_2 via leakage (ratio of CO_2 efflux to the total C_i uptake) strongly influences the efficiency of their CCMs. Under low CO_2 conditions, the leakage is high due to low ambient CO_2 concentrations and high internal C_i accumulation that both cause an increasing CO_2 gradient from inside the cells to the outside. High leakage causes high energetic requirements of the CCM, that in turn may decrease its efficiency (Raven & Lucas 1985, Rost et al. 2006a,b). Raven et al. (1982) hypothesised that the degree of the CO_2 permeability of the plasma membrane could change in response to the carbon supply, hence the CO_2 permeability coefficient would decrease under low CO_2,

thereby enhancing the efficiency of the CCM. However, Sültemeyer and Rinast (1996) showed that the CO_2 permeability constants for plasma membranes isolated from low and high C_i acclimated cells of the green alga *Chlamydomonas reinhardtii* were similar. Leakage has been estimated by means of MIMS following the approach by Badger et al. (1994) in two diatom species (Burkhardt et al. 2001, Rost et al. 2006a). This approach, however, requires absence of any eCA activity, which was assured by the presence of dextran-bound sulfonamide. As outlined above, eCA has been suggested to play a potential role in C_i-recycling. Hence, the approach of Badger et al. (1994) is not suited to measure leakage in diatoms.

Information on leakage has been gained by data on carbon isotope fractionation as well as on the estimates of the fractional contribution of HCO_3^- to total C_i uptake (*see* Publication II for more details on the calculation). The calculated leakage has been found to differ between diatom species (Publication II, S. Trimborn, unpublished data). Note that the calculated leakage is based on the assumption that the cell consists of a single compartment, an assumption obviously not matching the real structure of eukaryotic cells. As pointed out by Schulz et al. (2007) any internal C_i cycling at the level of the chloroplast will further decrease the leakage and subsequently ε_p, therefore it may only serve as an approximation of the upper most values for leakage possible. Consequently, this should be taken in consideration in the following discussion. In comparison, the 'HCO_3^- user' *S. stellaris* exhibited much higher leakage under high pH acclimation (70%) than the 'CO_2 user' *N. navis-varingica* (20%). Moreover, for *S. costatum* and *Eucampia zodiacus*, with 80% HCO_3^- uptake contributing to net fixation, values for leakage were up to 75% at ambient pCO_2 while values of 50% leakage were calculated for *Thalassionema nitzschioides* and *Thalassiosira pseudonana*, where equal quantities of HCO_3^- and CO_2 contributed to photosynthesis (Publication III, S. Trimborn, unpublished data). Hence these findings indicate that 'HCO_3^- users' exhibit much higher leakage than 'CO_2 users'. Additionally, the apparent affinities for C_i were high in all investigated diatom species irrespective of the preferred carbon source (Publication II, Publication III). It can thus be concluded, that 'HCO_3^- users' are as sensitive as, or even more sensitive than 'CO_2 users' with regard to their pH/CO_2-dependence in photosynthesis and growth. Thus, 'HCO_3^- users' are not necessarily less sensitive to changes in carbonate chemistry than 'CO_2 users', an observation that might be related to differences in leakage. In view of the uncertainties in current leakage estimates and the potential significance of the process, future investigations should develop new approaches to explore the mechanisms behind reduced leakage.

Beside the important aspect of leakage, the operation of a C_4-like photosynthetic pathway might significantly enhance the photosynthetic productivity in marine diatoms under low CO_2 conditions (Reinfelder at al. 2000). While it is mostly accepted that *Thalassiosira weissflogii* relies on a C_4-like metabolism (Reinfelder et al. 2000, 2004, Morel et al. 2002, Roberts et al. 2007, McGinn & Morel 2008), there is still controversy about the operation of a C_4-like pathway in *T. pseudonana* (Granum et al. 2004, Roberts et al. 2007, McGinn & Morel 2008) and even increasing evidence against such a pathway in other diatoms (Cassar & Laws 2007, Publication III). Using the same ^{14}C-based assay as Reinfelder et al. (2000), the ratio of PEPC relative to RubisCO activity did not increase despite strongly reduced CO_2 concentrations in laboratory experiments with *P. tricornutum* (Cassar & Laws 2007), *T. pseudonana* (Publication III) and the three ecologically relevant diatom species *E. zodiacus*, *T. nitzschioides* and *S. costatum* (Publication III). This is consistent with observations from field studies with diatom-dominated phytoplankton assemblages (Tortell et al. 2006, Tortell et al. submitted). More evidence for C_3 metabolism in the species mentioned above is given by results on carbon isotope fractionation, which yielded high ε_p between 10 and 16‰, while low ε_p values close to 0‰ would be expected if a large part of C_i would be assimilated via PEPC prior to the fixation by RubisCO (Cassar & Laws 2007, Publication III). Additionally, the expression of PEPC and PEPCK genes in *T. pseudonana* was independent of the CO_2 supply (Granum et al. 2005, Roberts et al. 2007). While there is increasing evidence against C_4 in *T. pseudonana* (Granum et al. 2005, Roberts et al. 2007, Publication III), *P. tricornutm* (Cassar & Laws 2007) and the three ecologically relevant diatom species *E. zodiacus*, *T. nitzschioides* and *S. costatum (*Publication III), contrasting results are shown by McGinn & Morel (2008) postulating the prevalence of C_4 metabolism in *T. pseudonana* and *P. tricornutum* based on analysis of gene transcripts of PEPC and PEPCK and inhibitor studies of these enzymes. As the operation of the C_4-like pathway should significantly enhance the photosynthetic productivity of marine diatoms under low CO_2 (Reinfelder at al. 2000), one might expect that diatoms relying on C_4 metabolism would have higher photosynthetic rates under low CO_2 conditions than diatoms without such a pathway. In fact, under low CO_2 *S. costatum* (Rost et al. 2003), which operates C_3 metabolism (Publication III), photosynthesises as efficiently as *T. weissflogii* (Burkhardt et al. 2001), in which C_4 metabolism has been shown (Reinfelder et al. 2000, 2004, Morel et al. 2002). As shown by the low $K_{1/2}$ (CO_2) for photosynthesis of <1 μmol L^{-1} CO_2 for both diatom species under low CO_2 (36 μatm CO_2, Burkhardt et al. 2001, Rost et al. 2003), it is evident that they operate highly efficient CCMs that in turn allow to reach high photosynthetic rates. Hence, if an additional C_4 pathway

would play a primary role in photosynthesis, this pathway nevertheless provides no competitive advantage over classical CCMs in marine diatoms.

In contrast to other phytoplankton taxa (Rost et al. 2003, Rost et al. 2006b), marine diatoms operate highly efficient and regulated CCMs (Publication II, Publication III). This ability may provide a competitive advantage in coastal areas as well as under changing conditions as they occur during a bloom. As the acclimations were carried out under nutrient replete conditions (Publication II, Publication III) and bloom situations cause a drawdown of the nutrient concentration especially towards their end, the effect of nutrient limitation in combination with CO_2-related changes in seawater carbonate chemistry should be considered in future research. Moreover, the experiments were performed under a stable light environment (Publication II, Publication III). Future projects should also focus on experimental setups, in which CO_2 and light are altered in combination, because marine diatoms mainly form blooms in turbulent waters during early spring, i.e. highly variable light conditions (e.g. MacIntyre et al. 2000, Rost et al. 2006a, Lavaud et al. 2007). According to their occurrence in turbulent waters, it might be expected that diatoms are much more efficient in balancing growth requirements to maintain metabolic processes compared to phytoplankton groups that are mainly found in stratified waters (e.g. Rost et al. 2006a). Coccolithophores, for instance, mostly thrive in stratified waters when nutrient concentrations are relatively low. As pointed out by Rost et al. (2006a), owing to its inefficient CCM, coccolithophores like *E. huxleyi* may not be able to compete with diatoms under early spring conditions.

Coccolithophore blooms are mainly observed in late spring/early summer, when the water column becomes stratified. For coccolithophores, it has been speculated that the process of calcification is involved in photosynthetic carbon acquisition (Sikes et al. 1980) providing a competitive advantage under low CO_2 supply. Thereafter, the calcification process releases CO_2, thereby supporting photosynthetic carbon fixation. Evidence against this functional coupling between photosynthesis and calcification comes from the observation that rates of photosynthesis in the calcifying strain of *E. huxleyi* were as high as in a non-calcifying strain (Rost & Riebesell 2004). Additionally, C_i flux measurements showed that HCO_3^- uptake rates of the non-calcifying strain were even higher than in the calcifying strain indicating that HCO_3^- utilisation is not tied to calcification. To exclude strain-specific differences for these findings, experiments have been carried out where the degree of calcification of a calcifying strain of *E. huxleyi* has been changed via the calcium (Ca^{2+}) concentration while keeping the CO_2 supply constant (Publication I). It was demonstrated that photosynthesis in *E. huxleyi* remained constant when calcification ceased

(Herfort et al. 2004, Publication I). This finding is consistent with Paasche (1964) and Herfort et al. (2002) showing that photosynthesis is not affected when *E. huxleyi* cells are transferred to Ca^{2+}-free medium. Moreover, the contribution of HCO_3^- uptake to carbon net fixation was constant, independent of the Ca^{2+} treatment and hence the degree of calcification (Publication I). Additionally, the ^{13}C fractionation pattern did not differ despite large differences in the calcification rates in *E. huxleyi* (Publication I). Therefore, it is concluded that the CO_2/protons produced by calcite precipitation does not stimulate photosynthesis. The pH in the coccolith vesicle of *Coccolithus pelagicus* was up to 8.3 while the pH in the cytosol was measured to be about 7.0 (Anning et al. 1996). As a consequence of this large pH difference, the coccolith vesicle would rather act as a sink for CO_2 and thus DIC as opposed to supplying photosynthesis with additional C_i. Finally, considering that coccolithophores such as *E. huxleyi* are well below CO_2 saturation at present day CO_2 levels (Rost et al. 2003), the proposed functional coupling between photosynthesis and calcification would in any case operate very inefficiently.

5.2 FROM THE LABORATORY TO THE FIELD: OCEAN ACIDIFICATION AND ITS IMPACT ON THE ECOLOGY OF MARINE DIATOMS

In view of the ongoing acidification of the oceans (Wolf-Gladrow et al. 1999, Orr et al. 2005, IPCC report I 2007), differences in efficiency and regulation of CCMs bear important ecological implications. These have prompted various CO_2 manipulation experiments over the last two decades to try to understand the effects of high CO_2 on productivity and species composition of marine phytoplankton. The effects of pCO_2 on carbon acquisition and photosynthesis of phytoplankton have primarily been studied in numerous laboratory experiments undertaken with monospecific microalgal cultures (*see* Giordano et al. 2005 for a review). Only few studies related to field data could show that CO_2 potentially affects the taxonomic composition, primary productivity and/or growth of natural marine phytoplankton assemblages (Hein & Sand-Jensen 1997, Tortell et al. 2000, Tortell et al. 2002, Riebesell et al. 2007, Publication IV). During shipboard CO_2 manipulation experiments, elevated pCO_2 resulted in a higher primary productivity and phytoplankton growth as well as in a taxonomic shift towards larger chain-forming diatom species (Publication IV). Reasons for that might include the down-regulation of the CCM owing to an increased diffusive CO_2 uptake and/or reduced energetic costs of the CCM under

elevated pCO_2. This may further imply an increase in carbon fixation and an optimised allocation of other resources (Raven & Johnston 1991).

To examine the effect of high pCO_2 on the carbon acquisition in marine natural phytoplankton, commonly, the ^{14}C disequilibrium technique is applied because of its high sensitivity with respect to the naturally low cell densities (Tortell & Morel 2002, Cassar et al. 2004, Martin & Tortell 2006, Tortell et al. 2006, Tortell et al. submitted, Publication IV). These studies reported that HCO_3^- is the main C_i source in natural marine phytoplankton assemblages. In diatom-dominated (Tortell & Morel 2002) and mixed phytoplankton assemblages (Martin & Tortell 2006), high pCO_2 levels resulted in a decrease of both the relative contribution of HCO_3^- uptake and the maximum C_i transport rates. These results suggest that phytoplankton responded to high CO_2 by decreasing their relative HCO_3^- utilisation and their cellular C_i transport capacity. Two further studies showed only a decrease in the maximum C_i transport rates in various phytoplankton populations in response to high pCO_2 (Tortell et al. submitted, Publication IV). The latter might be ascribed to decreasing C_i transport capacity without altering the relative HCO_3^- utilisation. Overall, it is indicated that natural phytoplankton populations down-regulate their CCM under high pCO_2. This effect may be associated with a decreased leakage owing to a smaller CO_2 gradient from inside the cells to the outside (e.g. Raven & Lucas 1985, Rost et al. 2006a, b) as well as a decrease in cellular affinities for CO_2 and HCO_3^- (e.g. Publication II, Publication III). However, the application of the ^{14}C disequilibrium technique does not allow assessing this directly.

Using shipboard incubations with different levels of CO_2, measurements of C_i uptake kinetics were conducted for the first time on natural phytoplankton populations (*see* Publication IV for more details). In these assays, the ^{14}C incorporation has been determined in cells that have been transferred to an initially CO_2-free assay buffer, to which increasing amounts of ^{14}C spiked C_i have subsequently been added. Apparent C_i affinities were estimated to be ~350 μmol L^{-1} and 600 μmol L^{-1} C_i at low and high pCO_2, respectively, exhibiting 2-fold lower affinities of the C_i transport systems under high pCO_2 (Fig. 7, S. Trimborn unpublished data). By means of the ^{14}C disequilibrium technique, for the same population the fraction of HCO_3^- relative to net fixation was high, generally ~90%, while maximum C_i transport rates decreased by ~2-fold in the high relative to the low CO_2 treatment (Publication IV). Therefore, the down-regulation of the maximum C_i transport capacity (obtained in the ^{14}C disequilibrium assays) in the high pCO_2 incubations is likely to also reflect the decrease in affinities of the C_i uptake systems (obtained by the $14C_i$ kinetic assay) under these conditions. It can thus be concluded that the observed increase in

primary productivity and growth of the phytoplankton assemblage under high pCO_2 might be attributed to the down-regulation of the CCM activity (Publication IV).

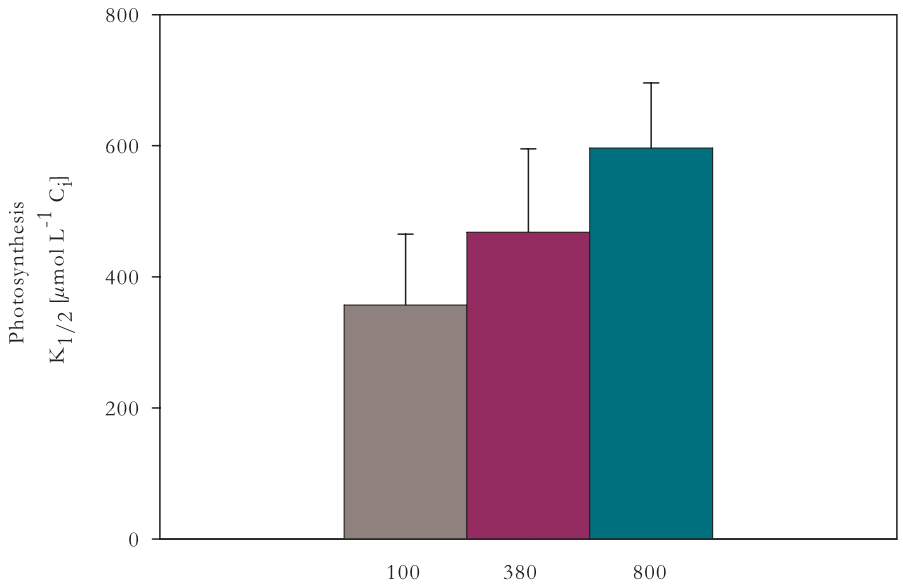


Fig. 7 $K_{1/2}$ values of photosynthesis for phytoplankton assemblages incubated at 100 μatm CO_2 (grey), 380 μatm CO_2 (purple) and 800 μatm CO_2 (cyan). Kinetic parameters were calculated from a Michaelis-Menten fit. Values for $K_{1/2}$ are given in μmol L^{-1} C_i.

In addition to the physiological changes, these shipboard experiments also revealed CO_2-dependent shifts in diatom species compositions. Within the phytoplankton assemblage, high pCO_2 promoted the growth of larger chain-forming diatom species (Publication IV). This finding is consistent with a previous field study by Tortell et al. (2002). Taking into account the available literature on CO_2-related changes of carbon acquisition in monospecific diatom cultures (e.g. Burkhardt et al. 2001, Rost et al. 2003, Publication II, Publication III), the lowest CO_2 affinity for photosynthesis was found for *S. stellaris*, a large centric diatom (7 μmol L^{-1}, Publication II). Careful extrapolation of the observed value to the carbonate chemistry of the ocean may indicate, in agreement to Publication IV, that especially large diatoms such as *S. stellaris* could benefit from the projected increase in CO_2. Based on the available data (Tortell et al. 2002, Publication II, Publication III, Publication IV), it can be concluded that marine diatoms in general, but large diatom species in particular, will benefit from increasing pCO_2 owing to an increased diffusive CO_2 uptake and/or reduced overall cost for C_i acquisition. The projected changes in seawater carbonate chemistry are likely to induce a species shift within diatoms, which

could translate into enhanced vertical fluxes of particulate organic carbon from surface waters to the deep ocean and representing a negative feedback on increased atmospheric CO_2.

The presented observations from laboratory and field experiments in marine diatoms highlight that the comparison of C_i uptake by both approaches can provide a better understanding of the physiological behaviour of phytoplankton populations in the oceans. Future projects should focus on the optimisation of the presented $^{14}C_i$ kinetic assay in the way that it allows an estimation of uptake kinetics for individual carbon sources. It is emphasised here that information on C_i uptake kinetics may help to gain a better understanding of the CO_2 sensitivity of marine phytoplankton. The presented $^{14}C_i$ kinetic assay could, for instance, be applied on size-fractionated phytoplankton assemblages. The information of differences in carbon acquisition between larger and smaller cells could help to predict the potential effect of increasing pCO_2 on future phytoplankton assemblages.

5.3 PERSPECTIVES FOR FUTURE RESEARCH

The results of this thesis provide new information on carbon acquisition in marine diatoms and coccolithophores. Several questions for future research arise from the findings presented here. As pointed out in Publication II and III strong species-specific differences in carbon acquisition exist within the group of diatoms. Future research should focus on species-specific responses by performing experiments with a large number of species. For example, it would be important to test whether the finding that calcification is not involved in the photosynthetic carbon acquisition in the coccolithophore *E. huxleyi* (Publication I) also applies to other representative coccolithophores.

The highly efficient and regulated CCMs observed in the investigated diatoms (Publication II, Publication III) may be related to their occurrence in coastal areas. Future experiments should examine whether the high CCM capacity is a general feature of diatoms by testing more oceanic species. For numerous diatom species a high plasticity in terms of the preferred carbon source as well as CO_2-dependent affinity changes in the C_i uptake systems could be shown (Publication II, Publication III). The question that needs to be addressed is whether the C_i uptake systems are post-translationally regulated rather than at the transcriptional level. To this end, a time course of the kinetic changes of the CO_2 and HCO_3^- uptake systems during transfer from high CO_2 to low CO_2 levels would be suitable. As the molecular and biochemical processes involved in the induction of high affinity uptake

systems are poorly understood, the analysis of gene transcripts and protein expression would provide valuable information.

The role of eCA in C_i recycling mechanisms (Publication II) needs to be investigated in more detail. The prevalence of a HCO_3^- transporter/CA complex could be elucidated by analysis of gene transcripts and protein abundances. By means of qPCR assays, it could be examined whether the genes of this complex would be up-regulated with decreasing CO_2. Moreover, inhibitor studies (e.g. 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid, vanadate, dextran-bound sulfonamide) could help to unravel the importance of eCA in carbon acquisition. For instance, by means of MIMS (Publication IV), the effect of the addition of dextran-bound sulfonamide, an inhibitor which is known to inhibit eCA, can be examined under different pH levels thus providing information on the role of eCA in photosynthetic carbon acquisition.

Publication II demonstrated that 'HCO_3^- users' are as sensitive as 'CO_2 users' with regard to their pH/CO_2-dependence of growth. It is believed that HCO_3^- uptake requires more energy than the active uptake of CO_2 implying higher energetic costs for 'HCO_3^- users'. Indirect indications therefore might be gained by experiments where cells are grown under saturating and limiting light conditions. The experiments could shed light on whether HCO_3^- is the preferred carbon source under high irradiances while CO_2 should be preferred under low irradiances due to energetic reasons. Moreover, as leakage might account for species-specific sensitivity to pH/CO_2-dependence of growth, the development of new MIMS approaches to determine leakage is required. The application of C_i flux measurements in combination with carbon isotope fractionation data can provide additional information on leakage (e.g. Publication II), but is based on several assumptions. Estimates on the C_i fluxes on the chloroplast level may improve this approach (*see* Schulz et al. 2007). The determination of leakage might also be important with respect to the evaluation whether diatom species operate a C_3 or a C_4 metabolism. One might expect that diatoms relying on C_4-like pathway should have a lower leakage than cells operating a C_3 metabolism.

A significant increase in primary productivity, growth and a change of the species composition of phytoplankton assemblages in the Ross Sea (Antarctica) has been observed under high pCO_2 (Publication IV). While CO_2 potentially influences marine phytoplankton structure and growth, other environmental factors such as light and iron availability also represent crucial factors in controlling the growth of marine phytoplankton in the Southern Ocean. Future research should examine the response of these environmental factors on ecologically relevant phytoplankton species of the Southern Ocean, since changes in

phytoplankton productivity in this region can have a strong impact on the carbon cycle in the future.

6 References

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Erklärung

Hiermit erkläre ich, das ich die Arbeit mit dem Titel:

'INORGANIC CARBON ACQUISITION OF MARINE PHYTOPLANKTON WITH EMPHASIS ON SELECTED DIATOM SPECIES'

selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit eidesstattlich, dass es sich bei den von mir abgebenen Arbeiten um 3 identische Exemplare handelt.

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Unterschrift