Responses of selected species of marine phytoplankton to increasing carbon dioxide and light

Dissertation

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To Earth…

Living beings do not merely exist in a snapshot,

but travel in a continuous line where they acclimate, evolve and die!

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1. Summary

Atmospheric carbon dioxide (CO_2) concentrations have been increasing since the industrial revolution and are expected to almost triple from pre-industrial values by the year 2100. CO_2 enters the ocean by atmosphere-surface ocean gas exchange, decreasing carbonate ion (CO_3^{2-}) concentrations and pH (ocean acidification). Additionally, the rise of CO_2 concentrations and other green-house gases in the atmosphere, increase global average temperatures in the air and, consequently, in the surface ocean. This strengthens thermal stratification, decreasing mixed layer depth and changing light availability. The overarching goal of this thesis was to investigate the effects of global change, namely of increasing CO_2 and light, on selected species of marine phytoplankton (cyanobacteria, coccolithophores and diatoms). At first, the focus was put on investigating the response of the evolutionarily oldest phytoplankton group (cyanobacteria) to changing CO_2 concentrations. From further work with cyanobacteria during mesocosm experiments in the Baltic Sea emerged the idea of determining the time necessary for phytoplankton cells to acclimate to the changed conditions. To assess this question the best studied phytoplankton species in the context of changing carbonate chemistry, *Emiliania huxleyi*, was chosen. Other cellular adjustments of high interest in a future ocean are those to sudden light variation, since organisms might be exposed to an average higher light intensity and more frequent high light events. It could be possible that diatoms such as *Phaeodactylum tricornutum* and coccolithophores like *Emiliania huxleyi* differ in their ability to cope with these changes.

Nitrogen fixing cyanobacteria (diazotrophs) are responsible for the input of new nitrogen into large areas of the ocean. Until the beginning of this thesis it was unknown whether and how they would respond to the expected changes in the ocean's carbonate chemistry. The important non-heterocystous diazotroph *Trichodesmium* strongly responded to rising CO_2 from 140 to 750 µatm, increasing cell division rate, nitrogen fixation rate per unit of phosphorus utilization and carbon fixation (publication I). In the heterocystous *Nodularia spumigena* (co-authored manuscript IV) and *Anabaena sp.* (co-authored manuscript V), however, nitrogen fixation was found to decrease with increasing CO_2, potentially resulting from decreasing pH and not the CO_2 concentration itself. Together these results hint to fundamental differences between heterocystous and non-heterocystous cyanobacteria. Hence, depending on their distribution, some regions could see increasing nitrogen fixation in the

future while others not. This could influence regional primary productivity and possibly carbon sequestration. Globally the distribution and abundance of non-heterocystous cyanobacteria in the oceans is more significant than that of heterocystous species, so the overall feedback will be determined by the former.

While the effect of increasing CO_2 concentrations on cyanobacteria only started to be analyzed very recently, other phytoplankton groups, especially coccolithophores, have already been considered for a longer period of time. Often studies were performed after the cells were pre-exposed to experimental CO_2 concentrations for about 9 to 12 generations. However, it is unknown how much time is actually required for cells to reach a new physiological "equilibrium" (acclimation). Hence, the frequently studied *Emiliania huxleyi* was exposed to abrupt variations in carbonate chemistry and its response followed over 26 hours. Cells acclimated within about 8 hours, increasing photosynthesis and decreasing calcification under elevated CO_2 concentrations, similar to cells pre-exposed to those conditions (manuscript II). If such a rapid acclimation is a general phenomenon within phytoplankton species it simplifies the interpretation of short-term results for several experimental setups such as mesocosm and ship-board incubations, since in these situations it is not feasible to pre-expose the communities to the experimental conditions.

As stated above, the expected rise of CO_2 concentrations might indirectly change the light supply for plankton. Phytoplankton species respond differently to dramatic (abrupt and strong) changes in light intensity, influencing their competitive fitness for a certain ecological niche. Exactly how cells photoacclimate to light changes and whether there are differences between species is still not completely clear. *Emiliania huxleyi* and *Phaeodactylum tricornutum* dissipated extra energy after an abrupt rise in light intensity as heat, fluorescence and photochemistry (manuscript III). However, there were differences between the species in both magnitude and timing of their individual responses. Additionally, the coccolithophore was found to use an additional dissipation valve, calcification. Species-specific responses to dramatic increases in light intensity as those found here might be important defining competitive fitness and therefore, community composition.

Results of this doctoral thesis point out the importance of the response of diazotrophs in marine feedbacks to global change. The strong increase in nitrogen fixation with rising CO_2 observed for the globally important *Trichodesmium* might provide a negative feedback, depending on the magnitude of the effect on other changing factors, such as temperature and

light. Moreover, changes in light intensity might influence community composition due to species-specific differences in response time, with potential consequences for the biological carbon pump.

Zusammenfassung

Die Konzentration von Kohlendioxid (CO_2) in der Atmosphäre steigt seit Beginn der industriellen Revolution kontinuierlich an und wird sich Prognosen zu Folge bis zum Jahr 2100 fast verdreifachen. Durch Gasaustausch zwischen Atmosphäre und Ozean gelangt CO_2 auch in den Oberflächenozean, wo es die Konzentrationen von Karbonationen (CO_3^{2-}) und den pH Wert verringert - ein Vorgang, der als Ozeanversauerung bezeichnet wird. Desweiteren steigen durch die Zunahme von CO_2 und anderen Treibhausgasen in der Atmosphäre auch globale Mitteltemperaturen über Land und im Oberflächenozean. Dies verstärkt die Dichteschichtung der oberen Wassermassen und reduziert damit die Tiefe der durchmischten Schicht und verändert somit die Verfügbarkeit von Licht. Ziel dieser Arbeit war es nun, die Auswirkungen des globalen Klimawandels, im Besonderen den Anstieg von CO_2 und Lichtverfügbarkeit, auf ausgewählte Arten des marinen Phytoplanktons (Cyanobakterien, Coccolithophoriden und Diatomeen) zu untersuchen. Dazu wurde als erstes der Einfluß von CO_2 auf die älteste der drei Gruppen, die Cyanobakterien, untersucht. In weiterführenden Mesokosmosexperimenten mit Cyanobakterien aus der Ostsee wurde dann die Idee geboren, die tatsächliche Zeit zu bestimmen, welche Phytoplankton braucht, um sich an neue, veränderte Bedinungen anzupassen. Für diese Fragestellung wurde eine der im Kontext von Ozeanversauerung am häufigsten untersuchten Arten, die Coccolithophoride *Emiliania huxleyi*, ausgewählt. Neben der Anpassung an die CO_2 Verfügbarkeit ist auch die an variable Lichtbedingungen von großer Bedeutung. Da im Ozean der Zukunft ausgeprägtere Dichteschichtungen und verringerte Durchmischungstiefen erwartet werden, wäre es denkbar, daß die Lebewesen in der euphotischen Zone höheren Lichtintensitäten und Lichtvariationen ausgesetzt würden. Darauf könnten etwa Diatomeen wie *Phaeodactylum tricornutum* und Coccolithophoriden wie *Emiliania huxleyi* unterschiedlich reagieren, da sie eventuell unterschiedlich gut mit diesen Änderungen zu Recht kommen.

In weiten Bereichen des Ozeans ist der von Cyanobakterien fixierte atmosphärische Stickstoff ein wichtiger Nährstoff im marinen Nahrungsnetz. Ob und wie diese Schlüsselorganismen auf zu erwartende Änderungen der Seewasserkarbonatchemie reagieren werden, war bis zu Beginn dieser Doktorarbeit jedoch vollkommen unklar. Hier konnte nun gezeigt werden, daß mit zunehmenden CO_2 Konzentrationen im Meerwasser (von 140 auf 750 µatm) die Zellteilungs-, und die Phosphat normalisierten Stickstoff und Kohlenstoff

Fixierungsraten eines wichtigen Stickstoff Fixierers, *Trichodesmium sp.*, deutlich ansteigen (Publikation I). In den Heterocysten bildenden Arten *Nodularia spumigena* (Co-Autorenschaft IV) und *Anabaena sp.* (Co-Autorenschaft V) hingegen nahmen die Stickstoffixierungsraten jedoch tendenziell ab. Es scheint also in dieser Hinsicht fundamentale Unterschiede zwischen Heterocysten bildenden Cyanobakterien und solchen ohne Heterocysten zu geben. Je nach Vorkommen könnte so in der Zukunft die Stickstoffixierung in einigen Regionen zunehmen, in anderen jedoch abnehmen. Das könnte sich dann auch auf die Fähigkeit des Ozeans Kohlenstoff aufzunehmen auswirken.

Während die CO_2 bedingten Effekte auf Stickstoff fixierende Cyanobakterien erst am Anfang ihrer Aufklärung stehen, so zieht eine andere Phytoplanktongruppe schon seit längerem reges Interesse auf sich. Zahlreiche Studien wurden an Coccolithophoriden durchgeführt, viele von ihnen an Kulturen, in denen die Zellen für etwa zwölf Generationen an die neuen CO_2 Bedingungen akklimiert wurden. Es ist jedoch unklar wie viel Zeit tatsächlich erforderlich ist, bis die Zellen ein neues physiologisches Equilibrium erreichen und somit akklimiert sind. Deshalb wurde die gut untersuchte Coccolithophoride *Emiliania huxleyi* einer abrupten Änderung der CO_2 Konzentration ausgestzt und ihre Reaktion darauf 26 Stunden lang verfolgt (Manuskript II). Nach nur etwa acht Stunden unter erhöhten CO_2 Bedingungen stieg die organische Kohlenstoffixierung an während zeitgleich die anorganische absank. Dies ist die typische Reaktion, welche für längere Zeit akklimierte Zellen charakteristisch ist. Daß Akklimationszeiten auch in anderen Phytoplanktonarten derart schnell verlaufen, ist eine wichtige Vorraussetzung für zahlreiche Experimente wie zum Beispiel solche mit natürlichen Planktongemeinschaften in Mesokosmen oder Deck-Inkubationen, bei denen es unmöglich ist, eine Akklimationsphase vor Beginn des eigentlichen Experimentes durchzuführen.

Neben direkten CO_2 Effekten wird Phytoplankton zukünftig auch indirekten ausgesetzt sein. Der prognostizierte Anstieg atmosphärischer CO_2 Konzentrationen wird als Folge wärmere Temperaturen im globalen Mittel mit sich bringen, welche die Dichteschichtung im Oberflächenozean verstärken und damit die durchmischte Schicht reduzieren könnten. Dies würde die Lichtverfügbarkeit für das marine Phytoplankton verändern. Unterschiedliche Arten sind unterschiedlich gut an variable Lichtverhältnisse angepaßt, was ihre kompetitive Fitness für ihre spezifische ökologische Nische beeinflußt. Wie genau Photoakklimation in Reaktion auf abrupte Änderungen in der Lichtintensität bei verschiedenen Arten abläuft ist

noch nicht eindeutig charakterisiert worden. Hier konnte nun gezeigt werden, daß die Coccolithophoride *Emiliania huxleyi* und die Diatomee *Phaeodactylum tricornutum* der zusätzlichen Energie einer abrupten Lichtintensitätserhöhung teilweise durch eine Reduktion von Lichtsammel- und verstärkter Produktion von Lichtschutzpigmenten begegneten (Manuskript III). Unterschiede zwischen den beiden untersuchten Arten zeigten sich hingegen hinsichtlich der Reaktionsgeschwindigkeit und der tatsächlichen Nutzung der zusätzlich verfügbaren Energie. Neben verstärkter Fixierung von Kohlenstoff in organische Materie bei beiden Arten, stieg auch die in anorganische durch Kalzifizierung bei *Emiliania huxleyi* an. Somit fungierte die Kalzifizierung als zusätzliche Energiesenke.

In Bezug auf marine Rückkopplungsprozess im Klimasystem Erde heben die hier erzielten Ergebnisse die Bedeutung von Stickstoff fixierenden Cyanobakterien deutlich hervor. Der prominente Astieg der Fixierungsraten der weit verbreiteten Art *Trichodesmium* mit zunehmenden CO_2 Konzentrationen könnte so einen negativen Rückkopplungsmeschanismus auf atmosphärisches CO_2 darstellen. Hier sind natürlich mögliche weitere Effekte, hervorgerufen durch Temperatur- und Lichtveränderungen, noch zu berücksichtigen. Schließlich könnten letztere die Zusammensetzung der Planktongemeinschaft durch Art spezifische Unterschiede in zellulären Reaktionszeiten beeinflussen. Es ist nicht auszuschließen, daß dies dann Auswirkungen auf die biologische Kohlenstoffpumpe hat.

Resumo

As concentrações de dióxido de carbono (CO_2) na atmosfera têm vindo a aumentar desde a revolução industrial, estando previsto que até ao ano 2100 quase tripliquem os valores existentes actualmente. O CO_2 é absorvido pela superfície do oceano por transferência gasosa. Isso induz um decréscimo da concentração de iões de carbonato (CO_3^{2-}) e pH, a que frequentemente se denomina por acidificação do oceano. O aumento do CO_2 na atmosfera em conjunto com outros gases de estufa tem vindo a aumentar a temperatura atmosférica média global. Isso eleva também a temperatura da superfície do oceano, intensificando a estratificação termal, diminuindo a profundidade de mistura e assim modificando a luz disponível. Esta tese de doutoramento teve como objectivo principal o estudo dos efeitos das mudanças globais, nomeadamente o aumento de CO_2 e luz, em espécies do fitoplâncton marinho pertencentes às cianobactérias, cocolitóforos e diatomáceas. Na primeira fase analisou-se o efeito do aumento da concentração de CO_2 nos fotoautotróficos mais antigos do planeta, as cianobactérias, mais concretamente no *Trichodesmium sp*.. Em posteriores experiências com mesocosmos no mar Báltico levantou-se a questão do tempo necessário para que as células se aclimatizem às novas condições. Para testar isso usámos uma espécie pertencente aos cocolitóforos, a *Emiliania huxleyi*, cuja resposta ao aumento de CO_2 está bem documentada. No oceano do futuro, as células terão também de se ambientar a outras mudanças, como seja o aumento da intensidade da luz média e de episódios de exposição a intensidades de luz elevadas. É possível que hajam diferenças na forma como as espécies reajem a essas mudanças, por exemplo entre a diatomácea *Phaeodactylum tricornutum* e o cocolitóforo *Emiliania huxleyi*.

As cianobactérias fixadoras de azoto são responsáveis pela introdução do azoto em áreas dos oceanos em que este é o factor limitante. Apesar da sua importância nos ciclos biogeoquímicos marinhos, a resposta destes organismos às esperadas mudanças globais ainda não tinha sido considerada até ao início desta tese. A primeira espécie a ser estudada foi *Trichodesmium sp.*. À medida que as concentrações de CO_2 aumentam de 140 para 750 µatm esta espécie aumentou a sua divisão celular, fixação de azoto em relação à utilização de fósforo e fixação de carbono (publicação I). Em dois estudos (*Nodularia spumigena* no manuscripto IV e *Anabaena sp.* no manuscripto V) executados posteriormente com cianobactérias que possuem células especializadas na fixação de azoto (heterocistos),

observou-se um decréscimo da fixação de azoto com o aumento de CO_2. A razão para essas diferenças pode dever-se a outros parametros que também variam quando se aumenta o CO_2, neste caso provavelmente o pH, serem mais evidentes do que a fertilização de CO_2. Estes resultados mostram que a resposta encontrada para as espécies sem heterocistos não pode ser generalizada para todas as cianobactérias fixadoras de azoto. Assim, de acordo com a resposta da espécie sem heterocistos, estes organismos podem vir a aumentar a produção primária em áreas com baixas concentrações de azoto, nomeadamente em zonas oligotróficas. Isso pode escassear as concentrações de fósforo nestas áreas e, assim, aumentar a sequestração de carbono no oceanos. Em oposição, as espécies com heterocistos poderão dar um reforço positivo ao clima. Contudo, no contexto global de fixação de azoto deverá haver um reforço negativo para o aumento das concentrações de CO_2, pois as espécies sem heterocistos são mais abundantes e têm uma área de distribuição maior do que as espécies com heterocistos.

As respostas dos cocolitóforos às previstas mudanças do clima já começaram a ser investigadas há mais tempo do que as cianobactérias. Frequentemente são efectuadas experiências após as culturas estarem aclimatizadas às condições que serão testadas. O tempo usado para esse efeito corresponde habitualmente entre 9 e 12 gerações. No entanto, este período é usado sem que se saiba realmente quanto tempo as células levam a aclimatizar, ou seja, a estabelecer um novo estado de equilíbrio fisiológico. Para perceber quanto tempo é necessário para isso, submetemos culturas de *Emiliania huxleyi* a um aumento abrupto das concentrações de CO_2. As células de *Emiliania huxleyi* atingiram um novo estado de equilíbrio, com um aumento da fotosíntese e um decrécimo da calcificação, em apenas 8 horas (manuscript II). Este novo estado de equilibrium assemelha-se a resultados obtidos com culturas previamente aclimatizadas. Caso esta resposta possa ser estrapulada para outras espécies fitoplanctónicas, poderá simplificar a interpretação dos resultados de estudos com comunidades naturais a bordo de navios ou em mesocosmos, pois nestas situações as experiências não podem decorrer durante muito tempo o que impede uma prévia aclimatização.

Como foi referido anteriormente, o aumento de CO_2 poderá influenciar, ainda que indirectamente, a intensidade da luz disponível aos organismos marinhos que habitam a superfície do oceano. As espécies do fitoplâncton não reagem todas da mesma forma a mudanças no fornecimento de luz, por esta razão algumas espécies ocorrem preferencialmente em zonas costerias e outras em zonas oceânicas. Embora a luz seja um parametro

determinante para a distribuição horizontal das comunidades fitoplanctónicas, ainda se desconhece a capacidade de fotoaclimatizar a mudanças abruptas da intensidade da luz. Quer a *Emiliania huxleyi* como o *Phaeodactylum tricornutum* dissipam parte do excesso de energia sob a forma de calor, fluorescência e fixação de carbono após serem expostos a um aumento da luz (manuscript III). No entanto, estas espécies variam na magnitude e rapidez da resposta. A diatomácea, *Phaeodactylum tricornutum*, aumenta a fixação de carbono mais rápidamente do que o cocolitóforo. Por outro lado, observou-se que o cocolitóforo pode usar a calcificação como uma "válvula de escape" para a energia excedente. Estas respostas específicas das espécies podem influenciar as relações inter-especificas e assim dar vantagem competitiva a certas espécies nas comunidades em que se encontram inseridas.

De acordo com estudos desenvolvidos nesta tese, tornou-se evidente a importância da resposta das cianobactérias fixadoras de azoto às mudanças globais. De facto, o aumento significativo da fixação de azoto com a elevação das concentrações de CO_2 por parte do *Trichodesimum* pode aumentar a produção primária e assim aumentar a absorção de CO_2 da superfície do oceano. A magnitude deste efeito irá depender de outros parametros que estão previstos mudar com a concentração de CO_2, por exemplo temperatura e luz. Nomeadamente, a variação de luz poderá influenciar a composição das comunidades fitoplanctónicas, pois as espécies têm diferentes tempos de reacção a essas mudanças. Isso poderá ter um efeito na bomba biológica de carbono.

2. Introduction

2.1. Changes in the past, present and future ocean

In the last 800 000 years atmospheric CO_2 concentrations fluctuated between 180 in colder glacial and 280 ppmv (parts per million per volume) in warmer interglacial periods (Figure 1). The warm periods were associated with dramatic changes in the ocean, namely melting of Northern Hemisphere ice sheets and rising sea-level. Although, it is unsure which mechanisms are responsible for variable atmospheric CO_2 concentrations, several hypotheses are under discussion [*Archer et al.*, 2000]. Lower atmospheric CO_2 during glacial periods could have been caused by stimulation of the organic carbon pump possibly due to increased macro [*Broecker & Henderson*, 1998; *McElroy*, 1983] or micro-nutrient availability [*Martin*, 1990], reduction in the intensity of the inorganic carbon pump [*Archer & Maier-Reimer*, 1994; *Sigman, McCorkle & Martin*, 1998] resulting from a reduced shelf area, or slowing down of deep water ventilation [*Toggweiler*, 1999].

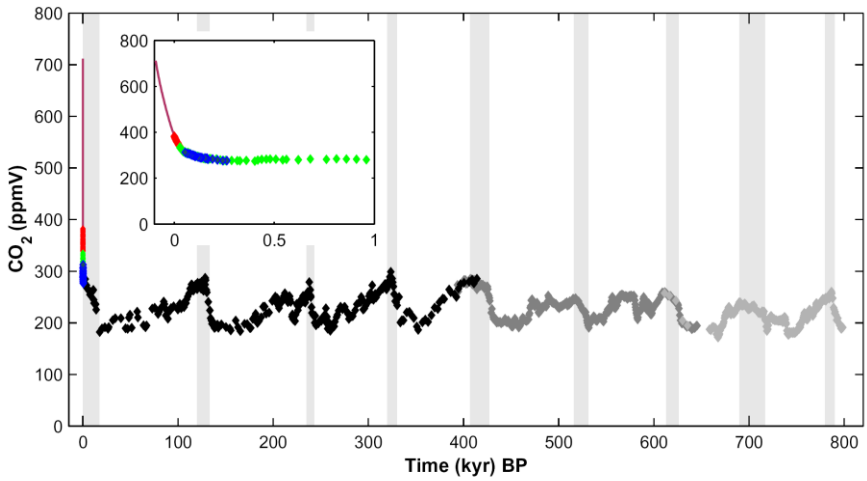

Fig. 1- CO_2 record dating back 800 000 years before present (BP) and projected until the year 2100. The graph combines data derived from measurements of trapped air in the Vostok and the Dome C Antarctic ice cores, actual atmospheric records and projected values for future years. Light grey symbols refer to the period between 800 and 650 kyr BP [*Lüthi et al.*, 2008], grey symbols between 650 and 390 kyr BP [*Monnin et al.*, 2004], black symbols between 414 and 2.3 kyr BP [*Petit et al.*, 1999], green symbols between 1006 A.D. to 1978 A.D. [*Etheridge et al.*, 1998], blue symbols between 1744 and 1953 [*Neftel et al.*, 1994], red symbols between 1980 and 2007 [Manoa Loa annual mean until 2008] and red line between 1765 and 2100 [*IS92a scenario, IPCC* 2007]. Grey vertical bands correspond to the start of warm periods, so called inter-glacial periods (based on *Lisiecki & Raymo*, 2005 and *Lüthi et al.*, 2008).

During glacial / interglacial transitions, CO_2 was basically shifted between two reservoirs, atmosphere and ocean. With the beginning of the industrial revolution, however, additional carbon from an extremely slowly overturning reservoir is being injected into these pools. The burning of fossil fuels, changes in land use and deforestation increased the atmospheric CO_2 concentrations from about 280 µatm to 380 µatm at present day, reaching values higher than those in the last 2 million years [*Hönisch et al.*, 2009]. More importantly, atmospheric CO_2 concentration is expected to continue to increase at unprecedented rates for a "business as usual" scenario (IS92a), reaching about 700 µatm by the year 2100, therefore almost tripling the pre-industrial values [*IPCC*, 2007].

While in the glacial / interglacial periods processes in the ocean were most likely causing atmospheric changes in CO_2, presently it is the atmosphere which dictates the changes in the ocean instead. Indeed, the ocean has taken up about one third of the anthropogenic CO_2 emissions since the industrial revolution [*Sabine et al.*, 2004]. The absorption of CO_2 changes seawater chemistry (see section 2.1.), namely decreasing oceanic pH (ocean acidification). Ongoing ocean acidification has already lead to a 0.1 pH unit decrease since the beginning of the industrial era [*Caldeira & Wickett*, 2005; *Orr et al.*, 2005].

The rise of CO_2 and other greenhouse gases such as methane (CH_4) and nitrous oxide (N_2O) in the atmosphere are thought to be responsible for increasing global average temperatures since mid-20th century [*IPCC*, 2007]. This is expected to drive melting of continental ice sheets and sea ice, thereby decreasing ice-albedo and the proportion of solar radiation that is reflected by Earth's surface. As a consequence, a higher amount of solar radiation is absorbed, reinforcing increasing temperatures in a positive feedback loop [*IPCC*, 2007]. Independent of the future CO_2 emission scenario, a doubling of atmospheric CO_2 concentrations alone would further increase global average temperature by 2 to 4.5 ºC [*IPCC*, 2007]. As a consequence, surface ocean temperatures will continue to increase, establishing a stronger thermal stratification and decreasing mixed layer depth. This might increase the barrier for upwelling of nutrient-rich deeper waters and change light supply. Light availability for phytoplankton might also vary in the future due to changes in cloud cover [*Sarmiento et al.*, 2004]. While the decrease of the mixed layer depth may have a positive effect on primary production in areas such as high latitudes where light is an important limiting factor, at lower latitudes there might be a negative effect due to decreased macro-nutrient availability [*Sarmiento et al.*, 2004]. In any case a

decreased mixed layer depth may favour species that perform better at overall higher light intensities (for information about photosynthesis and photoprotection see section 2.4.).

Additionally, human activities are perturbing also the nitrogen cycle by increasing nitrogen concentrations for instance as N_2O due to agriculture (main factor), industry and combustion of fossil fuels (Galloway et al., 2008). Nitrogen is washed into many coastal environments for instance through rivers and wastewater, but also to oceanic regions by atmospheric transport and deposition [*Duce et al.*, 2008]. Total deposition of anthropogenic nitrogen to the ocean increased from about 5.7 in the 1860 to 54 Tg N $year^{-1}$ in 2000 [*Duce et al.*, 2008]. This dramatic increase of anthropogenic nitrogen together with nitrogen fixation are responsible for net oceanic input in the open ocean [*Duce et al.*, 2008] and might be important in increasing primary production if the supply of other nutrients is available (see section 2.3.2).

2.2. Effects of increasing atmospheric CO_2 on the carbonate system of seawater

The ocean plays an important part in mitigating the rise of atmospheric CO_2 concentrations since the industrial revolution. CO_2 concentrations in the atmosphere equilibrate with surface ocean CO_2 by air-sea gas exchanges as

$$CO_2\,(g) \rightleftharpoons CO_2\,(aq) \qquad (1)$$

where the expressions (g) and (aq) denote gaseous and aqueous, respectively. Changes in surface ocean CO_2 concentrations have consequences for the other species of the carbonate system (see equilibrium reactions below and for details *Zeebe & Wolf-Gladrow*, 2001). As CO_2 increases it combines with water molecules forming carbonic acid (H_2CO_3). Being relatively instable (equation 2), H_2CO_3 dissociates into bicarbonate (HCO_3^-) and protons (H^+) (equation 3) which also combine with carbonate ions (CO_3^{2-}) (equation 5), further increasing HCO_3^- concentrations. H_2CO_3 exists in much lower concentrations then CO_2 and is difficult to separate from CO_2 analytically [*Dickson, Sabine & Christian*, 2007]. Thus, CO_2^* (equation 4) in water is conveniently defined as the sum of CO_2 and H_2CO_3.

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \quad (2)$$

$$H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \quad (3)$$

$$CO_2^* \overset{K_1}{\rightleftharpoons} H^+ + HCO_3^- \quad (4)$$

$$HCO_3^- \overset{K_2}{\rightleftharpoons} H^+ + CO_3^{2-} \quad (5)$$

The constants describing equilibrium conditions between CO_2^*, HCO_3^- and CO_3^{2-}, K_1 and K_2, depend on salinity, temperature and pressure of seawater. Another important equilibrium is that between the two CO_2 phases (gaseous and aqueous), described by K_0 also depend on salinity, temperature and pressure of seawater (equation 6).

$$[CO_2^*] = K_0 \text{ x } f(CO_2) \quad (6)$$

Here $[CO_2^*]$ is the concentration of CO_2^*. CO_2 fugacity $f(CO_2)$ describes the fugacity of CO_2 determined in the water phase in equilibrium with the gas phase at a known temperature and pressure. This takes into account the non-ideal gas behaviour of CO_2, being therefore slightly different from partial pressure values.

All carbon species in seawater are referred to as dissolved inorganic carbon (DIC) as

$$DIC = [HCO_3^-] + [CO_3^{2-}] + [CO_2^*] \quad (7)$$

At typical present day surface ocean pH values, less than 1% of DIC is in the form of CO_2^*, about 9 % as CO_3^{2-} and approximately 90 % as HCO_3^-. When the concentration of one of the components changes it causes a shift in the concentrations of the others, influencing pH (Figure 2).

The connecting element in the carbonate chemistry equilibrium reactions is the H^+ concentration. The concentration of hydrogen ions, the pH, in seawater is defined on the

total or free scale ($[H^+]_f$), being the difference that the total scale takes also into account bisulfate ion concentrations (HSO_4^-).

$$pH_f = - \log [H^+] \quad (8)$$

$$pH_T = - \log ([H^+] + [HSO_4^-]) \quad (9)$$

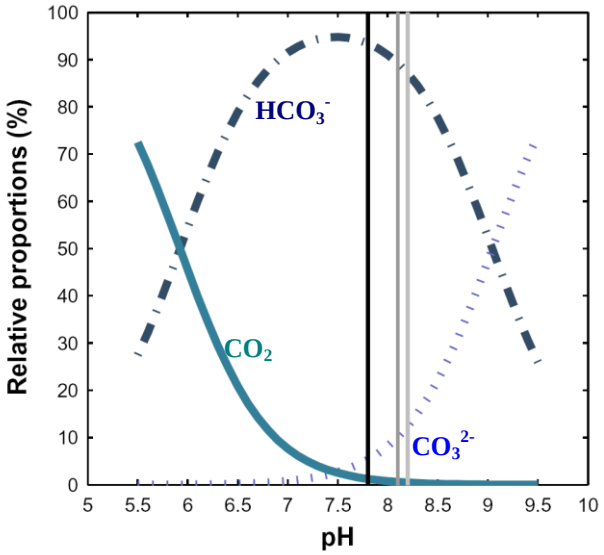

Fig. 2- Relative proportion of CO_2, HCO_3^- and CO_3^{2-} with respect to the total dissolved inorganic carbon pool at varying pH. The vertical lines denote typical seawater pH of the pre-industrial era (lighter grey), of present day (darker grey) and that expected for the year 2100 (black).

Another important parameter of the carbonate system is total alkalinity (TA). TA is defined as "…the number of moles of hydrogen ion equivalent to the excess of proton acceptors (bases formed from weak acids with a dissociation constant $K \leq 10^{-4.5}$ at 25ºC and zero ionic strength) over proton donors (acids with $K > 10^{-4.5}$) in 1 kilogram of sample." [*DOE*, 1994].

$$TA = [HCO_3^-] + 2[CO_3^{2-}] + [B(OH)_4^-] + [OH^-] + [HPO_4^{2-}] + 2[PO_4^{3-}] + [SiO(OH)_3^-] + [NH_3] + [HS^-] + \ldots - [H^+] - [HSO_4^-] - [HF] - [H_3PO4] - \ldots \quad (10)$$

Some species exist in very low concentrations and are therefore usually not included in the definition of TA (represented by "…").

Determination of two of the measurable parameters (pH, TA, DIC or $f(CO_2)$) allow to calculate the concentrations of all the remaining others (HCO_3^-, CO_3^{2-} and so on) from the stoichiometric equilibrium constants K_1, K_2 and K_0. Moreover, it is also possible to describe the calcium carbonate ($CaCO_3$) saturation state of seawater (Ω). This parameter determines whether $CaCO_3$ is a stable compound or starts to dissolve, therefore influencing the marine carbon cycle and controlling the CO_2 concentrations in the atmosphere (see section 2.3.1). The expected increase in CO_2 and related decreasing CO_3^{2-} ion concentrations lower $CaCO_3$ saturation state, according to the product of the calcium (Ca^{2+}) and CO_3^{2-} concentrations divided by the salinity, temperature and pressure dependent solubility product (equation 11).

$$K'sp = [Ca^{2+}]_{sat} \times [CO_3^{2-}]_{sat} \quad (11)$$

where $[Ca^{2+}]_{sat}$ and $[CO_3^{2-}]_{sat}$ refer to the ion concentrations in a seawater solution saturated with $CaCO_3$. Thus, the saturation state of seawater in relation to calcite and aragonite are expressed as

$$\Omega_{arag} = [Ca^{2+}] \times [CO_3^{2-}] / K'sp_{arag} \quad (12)$$

$$\Omega_{cal} = [Ca^{2+}] \times [CO_3^{2-}] / K'sp_{cal} \quad (13)$$

Calcifying organisms form $CaCO_3$ structures that depending on their crystal structure are composed of aragonite or calcite. In the open ocean, calcite is mostly produced by coccolithophores and foraminifera and aragonite by pteropodes. With ocean acidification the crystal structure becomes important since aragonite is more soluble than calcite under equal salinity, temperature and pressure.

The calcium concentration is usually considered to be conservative in relation to salinity. In certain areas, highly influenced by river input, the salinity / Ca^{2+} relation may however vary. When Ω is superior to 1 the seawater is supersaturated (calcification can occur) and if inferior to 1 it is undersaturated (dissolution occurs).

Biological activity changes the carbonate system of seawater in several ways (Table I). Processes such as production of organic compounds by carbon fixation (organic carbon

fixation, mostly photosynthesis), nutrient uptake, silicification, calcification and respiration may impact either DIC and / or TA, changing the carbonate chemistry speciation. Indeed, photosynthesis decreases DIC at constant TA (see photosynthesis equation in section 2.4.) while phosphate (PO_4^{3-}) uptake increases TA, but not DIC. The effects of PO_4^{3-} uptake on the carbonate chemistry occur because the reduction of TA by two units (1 µmol / kg of PO_4^{3-} equals 2 µmol / kg of TA, see equation 10) is reversed by an increase of three units in TA by the uptake of three H^+ which are necessary to maintain a constant membrane potential, counterbalancing the three negative charges of the molecule PO_4^{3-}. This increases pH, which in turn decreases CO_2 and HCO_3^- and increases CO_3^{2-} (see figure 2). Nitrate uptake (NO_3^-), similarly to PO_4^{3-}, decreases H^+ concentrations as well, decreasing CO_2 and HCO_3^- and increasing CO_3^{2-}. Silicification does not influence carbonate chemistry, as it is evident from equation 10.

Table I- Changes in the carbonate chemistry speciation by various physiological processes under typical environmental conditions (18ºC, 35 salinity, 2300µmol of TA and 2100µmol DIC).

Process	CO_2	HCO_3^-	CO_3^{2-}	DIC	TA	pH
Organic carbon fixation	↓	↓	↑	↓	–	↑
Nutrient uptake (PO_4^{3-}, NO_3^-)	↓	↓	↑	–	↑	↑
Silicification	–	–	–	–	–	–
Calcification	↑	↓	↓	↓	↓	↓
Nitrogen fixation	–	–	–	–	–	–
Respiration	↑	↑	↓	↑	–	↓

Calcification decreases both DIC and TA in a 1 to 2 relation, because CO_3^{2-} is incorporated into the calcium carbonate structures (see equation 10 and 16). Nitrogen fixation takes up molecular nitrogen and does not change the carbonate chemistry. When the organic carbon compounds are respired CO_2 is released increasing DIC, with no changes in TA, decreasing pH and CO_3^{2-} and increasing HCO_3^-.

The relative strength of these processes in the ocean influences biogeochemical element cycling.

2.3. Phytoplankton importance for the marine carbon and nitrogen cycles

There is a tight relation between Earth's climate and biosphere. Changes in environmental conditions influence organisms' physiology affecting biogeochemical cycles and feeding back to climate. Marine phytoplankton (microscopic photoautotrophs) is one of the key players in the marine carbon, nitrogen, silica, phosphorous and iron cycles.

2.3.1. Carbon cycle

Most carbon enters the oceanic realm in the form of CO_2 and coastal areas due to river input as HCO_3^-. CO_2 exchanges between atmosphere and surface ocean as its partial pressure establishes equilibrium. Atmospheric CO_2 is transported to depth either through the physical or the biological carbon pump. The physical pump is driven by water temperature changes. As water masses cool and become denser while ice forms at high latitudes (exception made to the North Pacific) they sink. This cold water has high CO_2 solubility, therefore transporting increased amounts of DIC to depth. On the contrary, warm surface water masses have lower CO_2 solubility and consequently also lower DIC concentrations.

At present, because of the high CO_2 concentrations in the atmosphere, the air-sea exchange causes a vertically decreasing gradient of anthropogenic CO_2 from the surface ocean to depth. Horizontal distribution of anthropogenic CO_2 concentrations is also not homogeneous, having the North Atlantic the highest vertically integrated values [*Sabine et al.*, 2004]. Vertical penetration of anthropogenic CO_2 is stronger at high latitudes (with exception of the North Pacific) due to deep water formation, while anthropogenic CO_2 concentration at the surface ocean is highest at low latitudes (warm tropical and subtropical waters) because of seawater buffer capacity governed by the Revelle factor. The Revelle factor "describes how partial pressure of CO_2 in seawater (pCO_2) changes for a given DIC." [*Sabine et al.*, 2004]. Because the Revelle factor is inversely correlated to the uptake capacity of the ocean in a certain region, a low Revelle factor corresponds to a high buffer or uptake potential.

Additionally, CO_2 uptake is also influenced by the biological carbon pump which can be subdivided into the organic carbon and the inorganic or carbonate pump. In the organic

carbon pump, photoautotrophs temporarily draw down CO_2 from the surface waters via photosynthesis which products are partly exported to the deep ocean (Figure 3). On the contrary, the carbonate pump, also called counter-pump, increases CO_2 at the surface (see equation 16), as coccolithophores, foraminifera and pteropodes build calcium carbonate structures which subsequently sink to the deep. Most of the organic matter produced is remineralized in the euphotic zone [*Falkowski*, 2005] via the microbial loop [*Azam*, 1989], the remaining fraction is exported to depth together with the inorganic part. From the total amount of carbon fixed per annum 5-10 % sinks in the central ocean basins [*Dugdale & Wilkerson*, 1992] and 50 % at high latitudes and in nutrient-rich areas, mostly due to diatom blooms [*Bienfang*, 1992]. In the deep, particulate organic matter is remineralized, thereby increasing DIC. Deep waters rich in CO_2 and nutrients come closer to the surface either through wind driven upwelling on timescales of decades to centuries or due to overturning of the global ocean in periods of about 1000 years [*Broecker & Peng*, 1982].

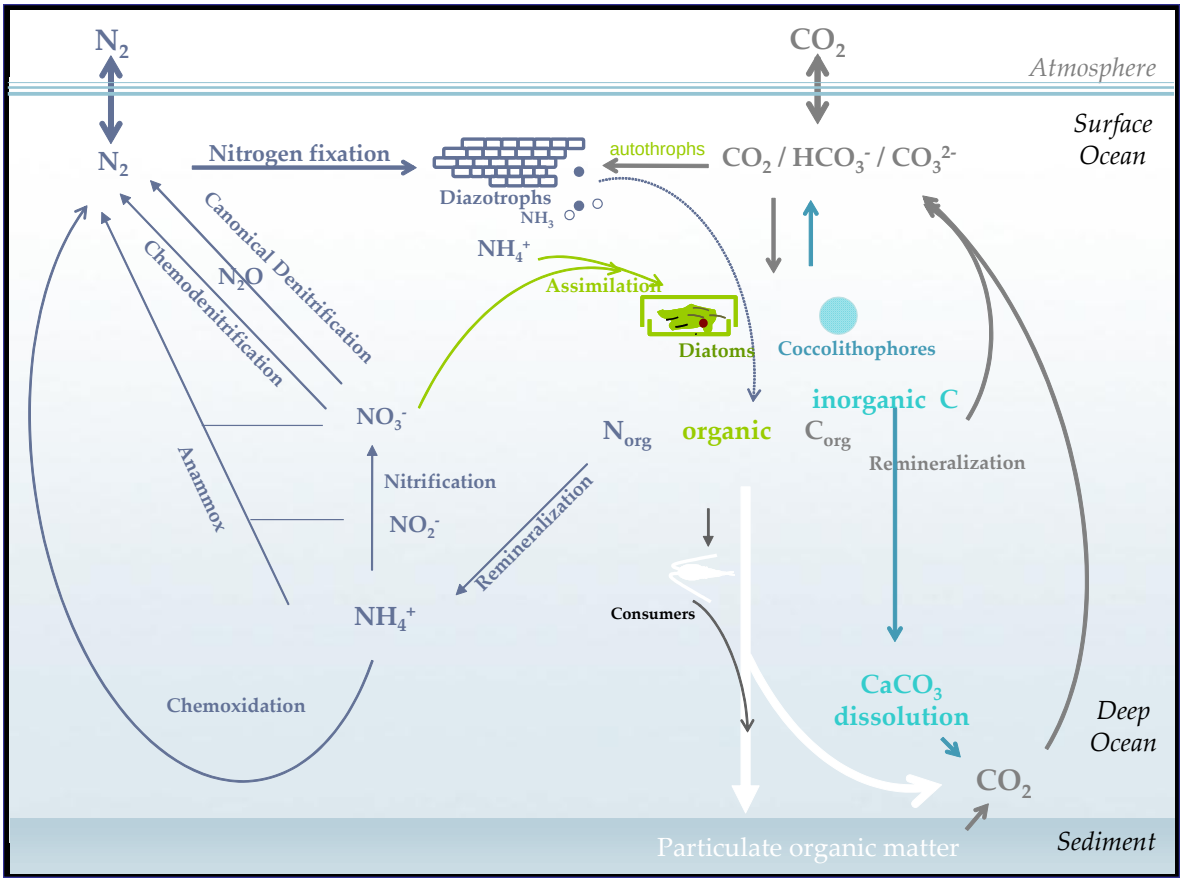

Fig. 3- Scheme of the marine nitrogen and carbon cycles. Nitrogen cycle (dark blue), organic carbon pump (white) and carbonate pump (light blue). Nitrogen cycle adapted from *Brandes et al.* [2007]. Note that denitrification, anammox, chemoxidation and nitrification may occur at different water column depths and in sediments.

The ratio between particulate inorganic carbon (PIC) to particulate organic carbon (POC) of the sinking matter is termed the rain ratio. The rain ratio influences the ocean-atmosphere fluxes of CO_2 [*Rost & Riebesell*, 2004]. When PIC / POC increases the ocean's capacity to sequester CO_2 decreases and vice-versa. It depends on one hand on calcification by coccolithophores, foraminifera and pteropodes and on the other hand on organic carbon fixation by phytoplankton, mostly diatoms. Hence, if physiological adjustments of phytoplankton to changes in the future ocean affect the rain ratio, there will be consequences for the atmospheric CO_2 uptake by the ocean.

While diatoms (organic carbon pump) and coccolithophores (organic carbon pump and counter-pump) have obvious roles in the biological pump, nitrogen fixing cyanobacteria are thought to have a smaller direct contribution because many species have positive buoyancy and are, therefore, thought to be mostly recycled in the microbial loop. However, there are still great uncertainties regarding this issue, for instance cyanobacteria perform programmed cell death which could be a vehicle of vertical transport of cyanobacterial "vesicles" and they occur as symbionts of diatoms. Furthermore, over longer time scales the addition of bioavailable nitrogen by these organisms impacts the global carbon cycle [*Falkowski*, 1997].

2.3.2. Nitrogen cycle

Input of nitrogen to the ocean occurs by nitrogen fixation, riverine and atmospheric deposition. The nitrogen cycle is driven by several key groups of organisms. N_2-fixing (diazotrophic) cyanobacteria provide the biological source of new nitrogen to the aquatic environment (see equation 15). Part of the fixed nitrogen is used by the cells and the rest often exuded (Figure 3). Taking *Trichodesmium* as an example, diazotrophs might exude fixed nitrogen as NH_4^+ (NH_3 formed from N_2 is a gas and reacts with water forming ammonium (NH_4^+)). [*Mulholland, Bronk & Capone*, 2004], dissolved organic nitrogen [*Glibert & Bronk*, 1994] and amino acids [*Capone, Ferrier & Carpenter*, 1994]. *Trichodesmium* is an important player in the nitrogen cycle, thought to account for more than half of the new production in parts of the oligotrophic tropical and subtropical oceans [*Capone et al.*, 2005; *Mahaffey, Michaels & Capone*, 2005], with estimations from direct measurements varying from 60 to 85 Tg N a^{-1} [*Galloway et al.*, 2004; *LaRoche & Breitbarth*, 2005; *Mahaffey, Michaels & Capone*, 2005]. However, unicellular nitrogen fixers have been found to be important contributors to diazotrophic community N_2 fixation in the North Pacific [*Montoya et al.*, 2004; *Zehr et al.*, 2001] and

Atlantic [*Langlois, LaRoche & Raab*, 2005], but their overall contribution remains unknown.

The fixed nitrogen at the surface ocean can be directly assimilated as NH_4^+ by phytoplankton and bacteria or taken up after being oxidized to nitrite (NO_2^-) and nitrate (NO_3^-) by the chemolithoautotrophic bacterial nitrification at deeper depths. Nitrification has two oxidation steps. First, oxidation of NH_4^+ to NO_2^- by *Nitrosomas* and then a second oxidation of NO_2^- to NO_3^- by *Nitrobacter*. NO_3^- can be reduced back to NH_4^+ in anoxic sediments through the so called dissimilatory nitrate reduction to ammonium (DNRA).

After nitrogen is built into the particulate organic nitrogen, it is either remineralized in the microbial loop and / or sinks to the deep, usually due to programmed cell death, grazing and / or viral attack. In the deep particulate organic nitrogen is decomposed mainly by aerobic oxidation resulting in the release of NH_3 by remineralization and NO_3^- by nitrification. Several processes reduce NO_3^- and NH_4^+ back to molecular nitrogen (N_2) by: 1) canonical denitrification, in which bacteria reduce NO_3^- to gaseous compounds (N_2O, N_2) under suboxic conditions; 2) denitrification of NO_3^- (chemodenitrification) or NH_4^+ (chemoxidation) with manganese species in oxic-anoxic interfaces [*Davidson & Seitzinger*, 2006; *Luther et al.*, 1997]; 3) Anamox (Anaerobic ammonium oxidation) in which NH_4^+ is oxidized by NO_2^- or NO_3^-, being converted into N_2 under suboxic or anoxic conditions; and 4) also oxidation of NH_4^+, but only with NO_2^- through a not strictly anaerobic process named oxygen-limited autotrophic nitrification – denitrification (OLAND).

Even though considerable progress has been made in understanding various aspects of the nitrogen cycle and its key players in the last decades, there are still many questions to unveil. For instance, how all these processes occur in time and space and whether the organisms involved are sensitive to climate change. Because most of the processes described above occur in the deep, they are not expected to be immediately affected by climate change in contrast to nitrogen fixation which occurs in the surface ocean. However, at depth bacteria might be indirectly affected for instance, if increasing CO_2 concentrations affect nitrogen fixers or other phytoplankton species and alter organic matter export. This could dramatically influence oxygen concentrations at deeper waters, extending oxygen minimum zones [*Oschlies et al.*, 2008]. Thus it is crucial to understand phytoplankton physiology and sensitivity to climate change.

2.4. Marine phytoplankton

Phytoplankton are microscopic photosynthetic organisms passively transported through the water column. While performing photosynthesis they use light as energy source for carbon fixation. This can be represented in the general reaction

$$2H_2X + CO_2 + light \xrightarrow{pigment} (CH_2O) + H_2O + 2X \quad (14)$$

where the compound X is an atom of oxygen (O_2) in most phytoplankton and an atom of sulphur in anaerobic bacteria [*Falkowski & Raven*, 1997]. Hence, in phytoplankton the antenna pigments capture light energy, split H_2O and use the resulting electron to produce energy in the electron chain. This energy can then be used to fix carbon dioxide (CO_2) by the enzyme Rubisco (ribulose-1,5-bisphosphate carboxylase / oxygenase) forming carbohydrates (CH_2O), water (H_2O) and oxygen (O_2) as by-products.

While transported upwards in the water column phytoplankton cells might be exposed to sudden light intensity increases in a matter of seconds [*MacIntyre, Kana & Geider*, 2000], increasing light absorption by chlorophyll (Chl) and accessory pigments beyond possible utilization. For that reason, cells have mechanisms that dissipate the occasional excess energy. Chlorophyll relaxes from its excited state through the production of reactive oxygen species, fluorescence, heat (non photochemical quenching - NPQ) and photochemistry [*Müller, Li & Niyogi*, 2001]. The production of O_2 reactive species can be damaging to the cells, but this is minimized by the other energy dissipation valves, NPQ and photochemistry. NPQ in phytoplankton is correlated with the xanthophyll cycle [*Bassi & Caffarri*, 2000; *Horton, Ruban & Walters*, 1996] and energy is dissipated following the increase of light intensity by de-epoxidizing diadinoxanthin to diatoxanthin [*Brunet, Casotti & Vantrepotte*, 2008].

In addition to CO_2 and light, phytoplankton needs macro (silica, nitrate and phosphate) and micro-nutrients (e.g. iron, zinc, copper) for growth. They can assimilate these nutrients as various compounds. Cyanobacteria, for instance can use urea, diazothrophs molecular nitrogen [*Flores & Herrero*, 2005] and some species even amino acids, especially glutamine and arginine [*Flores & Herrero*, 1994], but the preferred nitrogen source of cyanobacteria is ammonium (NH_4^+) [*Flores & Herrero*, 2005].

Phytoplankton generally occur unicellularly, either solitary or forming colonies [*Zeitzschel*, 1978]. Species can be organized in functional groups according to their

morphology, physiology and ecology [*Reynolds et al.*, 2002] or due to their biogeochemical role in elemental cycling. In this thesis four phytoplankton functional groups are briefly presented: cyanobacteria (prokariotes), coccolithophores (calcifying), dinoflagellates (build a teca) and diatoms (silicifiers) (Figure 4).

Cyanobacteria (Cyanophyta) are gram negative prokaryotes with an extra outer membrane of lipopolyssacharide and proteins. Moreover, they have a thinner peptidoglycan layer in their cell wall than gram positive bacteria. The pigments used in photosynthesis and as protection for excess light (photoprotection) are chlorophyll, carotenoids and phycoerythrins and in most species additionally phycobilisomes. Some cyanobacteria are able to fix molecular nitrogen (diazotrophs) into a biologically available form (see equation 15) and, therefore, provide a nitrogen source in areas of low nitrate concentration.

$$N_2 + 8H^+ + 8e^- + 16ATP \xrightarrow{Nitrogenase} 2NH_3 + H_2 + 16ADP + 16P_i \quad (15)$$

The enzyme responsible for breaking the strong triple bonds of N_2 (nitrogenase) fixes molecular nitrogen (N_2), utilizing protons (H^+), electrons (e^-), and adenosine triphosphate (ATP) forming ammonia (NH_3), adenosine diphosphate (ADP), hydrogen (H_2), and inorganic phosphorus (Pi). Nitrogenase consists of a nitrogenase reductase (Fe-protein) and a dinitrogenase (Fe-Mo-protein) [*Postgate*, 1982]. To avoid that the enzyme is irreversibly inhibited by O_2, an abundant molecule in seawater and produced by photosynthesis, cyanobacteria have developed various ways to minimize O_2 concentrations in the proximity of the enzyme. Some species separate carbon from nitrogen fixation in time, such as *Trichodesmium* and *Crocosphaera* while others do it spatially with specialized cells (heterocystous) like *Nodularia* and *Anabaena.* Nitrogen fixation can be inhibited by the presence of other nitrogen sources as well. Cyanobacteria can perform simple cell division, filament fragmentation and spores formation.

Fig. 4- Examples of organisms of each functional phytoplankton group. Scales from top down: 25µm; 1µm; 5µm; 5µm.

Dinoflagellates (Dinophyta) are eukaryotic cells, which build a multilayer teca and are a very diverse group which contain various pigments as chlorophyll *a*, c_2, β, β-carotene, dinoxanthin, diadinoxanthin and peridines [*Jeffrey, Mantoura & Wright*, 1997]. They possess two flagella. Dinoflagellates exist most of their life-time as haploids being the only diploid phase the zygote (haplont life-cycle).

Coccolithophores (Haptophyta) are eukaryotic cells, which build calcium carbonate scales, the coccoliths (see equation 16). These coccoliths are built internally and channelled out to the exterior of the cell membrane forming a coccosphere. Even though coccoliths function is still under debate as well as which form of dissolved inorganic carbon is used during calcification, it is known that calcium ions (Ca^{2+}) are bound to dissolved inorganic carbon, for example in the form of bicarbonate (HCO_3^-), forming calcium carbonate ($CaCO_3$), CO_2 and H_2O.

$$Ca^{2+} + 2HCO_3^- \longrightarrow CaCO_3 + CO_2 + H_2O \qquad (16)$$

As pigments they have chlorophyll *a*, c_1 *or* c_3, β, β-carotene, 19'-hexanoyloxyfucoxanthin, fucoxanthin, 19'-butanoyloxyfucoxanthin, dinoxanthin and diadinoxanthin [*Jeffrey, Mantoura & Wright*, 1997]. They possess two flagella during the haploid phase and one haptonema [*Sieburth*, 1979]. Coccolithophores occur both as haploids and diploids (haplo-diploid life-cycle), with distinct morphologies in the two life-cycle phases.

Diatoms (Bacillariophyta) are eukaryotic cells with the ability to built two external silica structures (valves) forming a frustule. As pigments for photosynthesis and photoprotection they have chlolophyll *a*, c_2, c_1 *or* c_3, β, β-carotene, fucoxanthin, diadinoxanthin and diatoxanthin [*Jeffrey, Mantoura & Wright*, 1997]. They do not possess flagella. Diatoms exist most of the time in the diploid phase, being the only haploid phase the gametes (diplont life-cycle).

Many of the phytoplankton group specific characteristics (such as pigment composition) can be explained by their evolution, as fruit of genetic information from both the host cell and engulfed cyanobacterium (see below).

2.5. Phytoplankton evolution

Earth exists for about 4.6 billion years. The presence of liquid water on this blue planet created the conditions for life to appear about 3.5 billions years ago. Another important step for the evolution of life as we know it was oxygen evolving photosynthesis [*Falkowski & Knoll*, 2007]. It is thought that the organisms responsible for the dramatic change in the atmosphere and ocean, by increasing O_2 concentration [*Anbar et al.*, 2007; *Farquhar, Bao & Thiemens*, 2000], belonged to the cyanophyta and appeared about 2.7 billion years ago, perhaps even earlier [*Buick*, 1992; *Rosing & Frei*, 2004]. In a peculiar way, this was the starting point of all existing phytoplankton species.

A coccoid cyanobacterium was engulfed in the first endosymbiosis by a eukaryotic cell with a mitochondrium (Figure 5) [*Taylor*, 1987] forming the primary symbiotic oxygenic eukaryote [*Falkowski et al.*, 2004]. It is still under debate the number of times that this endosymbioses occurred, but evidences point to only one [*Palmer*, 2003]. The symbiont kept information from both ancestors, but the characteristics that separate the main plastid lineages are related much more to the evolutionary history of the engulfed cell [*Falkowski et al.*, 2004]. This eukaryotic primary endosymbionts of both the red and green lineage (see Figure 5), but not glaucophytes suffered a second endosymbioses. Again, there is discussion whether this endosymbioses occurred once or several times [*Grzebyk et al.*, 2004; *Keeling et al.*, 2004]. However, the theory supporting a polyphyletic origin, with different host cells, seems to be strongly supported by comparative analysis of plastid genomes [*Grzebyk et al.*, 2003]. Within the red lineage ("red algae"), these distinct hosts gave rise to the functional groups presented above (coccolithophores, dinoflagellates and diatoms), with cells possessing chloroplasts with three to four membranes. Thus, even though oxygenic photosynthesis seems to have evolved only once in time, extant eukaryotic algae have polyphylogenetic origins [*Delwiche*, 1999; *Palmer*, 2003].

The extant dominant representatives of the red lineage, dinoflagellates and coccolithophores appeared in the fossil record during the Triassic [*Bralower, Bown & Siesser*, 1991], and diatoms more recently at the Permian-Triassic boundary [*Koositra et al.*, 2002]. While the oceans are predominantly inhabited by the red lineage, on land it is the green clade the dominant one.

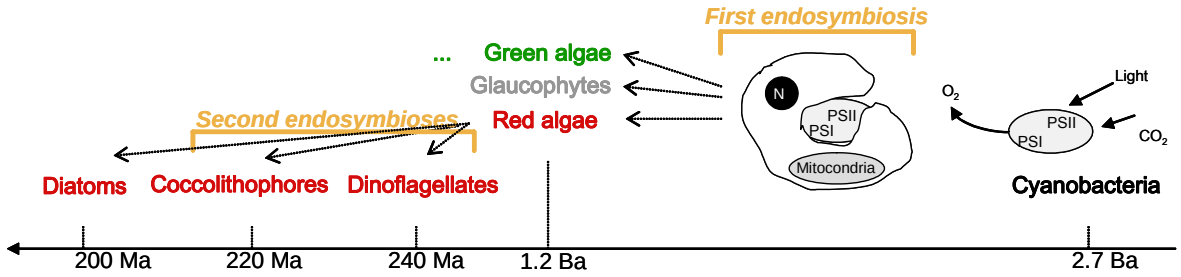

Fig. 5- Scheme of plastid engulfment and inheritance in phytoplankton. The emphasis was put on the red lineage, so the evolution of the green lineage was represented with "…". Green algae gave rise to land plants and, by secondary endosymbioses, to other phytoplankton species (not shown). Some dinoflagellates experienced a third endosymbiosis (not shown). While both dinoflagellates and coccolithophores can be followed back in the fossil record considerably well, diatom frustules tend to dissolve, making it harder to precisely date their appearance. N represents nucleus, PSI and PSII, photosystem I and II, respectively and, Ba and Ma for billion and million years ago. The scheme is based on *Falkowski et al.* [2004].

In essence, when considering present day diversity of species' physiology and responses to changing environments one should keep in mind their evolutionary history. Moreover, phytoplankton evolved at times of quite variable climate conditions, such as changing CO_2 concentrations.

2.6. Phytoplankton CO_2 concentrating mechanisms (CCMs)

Cyanobacteria appeared in an environment with high CO_2 concentrations (over 100 fold higher than pre-industrial values [*Badger & Price*, 2003]). Probably because of that, the enzyme responsible for organic carbon fixation (Rubisco) in all phytoplankton groups has relatively low affinity for CO_2. With the appearance of photoautothrophs CO_2 concentrations decreased and O_2 increased in the atmosphere, exerting selective pressure on the evolution of Rubisco's half saturation [*Raven & Beardall*, 2003]. However, even the Rubisco with the highest selectivity for CO_2 in relation to O_2 found in the most recent phytoplankton groups would still have low carboxylation and high oxygenation rates if they would depend merely on diffusive CO_2 supply [*Raven & Beardall*, 2003]. To compensate for this, phytoplankton evolved mechanisms to concentrate CO_2 (CCM) close to Rubisco [*Raven*, 1997], improving carboxylation [*Badger & Price*, 1992; *Kaplan & Reinhold*, 1999]. This can be achieved in several ways, either by: 1) active transport of HCO_3^- and / or CO_2 across membranes; 2) accumulation of HCO_3^- inside the cell or possibly fixed into a C_4 like compound (diatoms); 3) transport of HCO_3^- or CO_2 to a space

surrounded by membranes with low gas permeability where Rubisco is concentrated (carboxysoma in cyanobacteria and pyrenoids in most eukaryotic cells) and 4) presence of carbonic anhydrase (CA) which converts CO_2 into HCO_3^- and vice-versa, (re)establishing an equilibrium [*Moroney & Somanchi*, 1999]. Even though the detailed machinery used varies considerably, depending for instance on the Rubisco type, in all cases known the cells have to concentrate CO_2 against a gradient from the surrounding water to the cytoplasma. Moreover, studies done to date indicate that internal pH is generally lower than that of natural seawater, facilitating the efflux of CO_2. Thus, higher external CO_2 concentrations could reduce the efflux and thereby costs for operating the CCM (Figure 6).

Cyanobacteria are the oldest forms of life, having a Rubisco with lower affinity to CO_2 in relation to O_2 than other phytoplankton species. Hence, cyanobacteria might need higher CCM activity [*Badger & Price*, 2003]. In general, cyanobacteria have passive CO_2 uptake, followed by conversion of CO_2 to HCO_3^- [*Badger & Price*, 2003], probably in the thylakoid membranes in the proximity of the carboxysoma. In some cyanobacteria such as *Trichodesmium*, HCO_3^- is the carbonate species transported. Indeed a low affinity Na^+ / HCO_3^- symport is present [*Badger et al.*, 2006], likely dependent on an energy demanding Na^+ / H^+ antiport system [*Badger et al.*, 2006; *Giordano, Beardall & Raven*, 2005] and a low affinity CO_2 uptake complex dependent on reductive power [*Badger et al.*, 2006] (Figure 6).

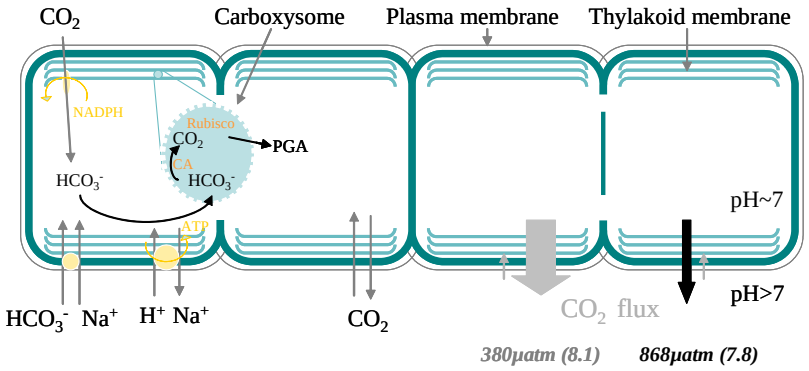

Fig. 6- Schematic diagram of the CO_2 concentration mechanisms in the cyanobacterium *Trichodesimum*. External elevated CO_2 concentrations can reduce the diffusive loss of CO_2 from the cell (represented by different thickness of the arrows) and / or increase the proportion of diffusive CO_2 uptake into the cell, resulting in a down-regulation of CCM activity. Coccolithophores and diatoms have additional membranes surrounding the chloroplast. Moreover, diatoms might have a C_4 mechanism which serves as an intermediate pool of captured CO_2. Adapted from *Badger et al.* [2006].

Rubisco of diatoms saturates at lower substrate concentrations and has increased selectivity for CO_2 in relation to O_2 compared to cyanobacteria [*Whitney, Graham & Andrews*, 2001]. Diatoms and coccolithophores take up both CO_2 and HCO_3^- [*Burkhardt et al.*, 2001; *Matsuda, Hara & Colman*, 2001; *Rost, Riebesell & Burkhardt*, 2003]. While the HCO_3^- uptake requires energy [*Gradmann & Boyd*, 1995] or reductive power, the transport of CO_2 might be passive or use energy [*Badger et al.*, 1998]. In diatoms a C_4 -like mechanism might function as a CCM by fixing part of the CO_2 taken up into an organic compound and delivering it to the chloroplast where it is transformed back to CO_2 near Rubisco [*Reinfelder, Kraepiel & Morel*, 2000].

While CCM's have been developed on geological time scales to compensate for reducing CO_2 availability in the environment they also serve as regulatory valves adjusting CO_2 supply with demand on shorter time scales. Indeed, CO_2 availability to the cells alters seasonally and during bloom events (Figure 7).

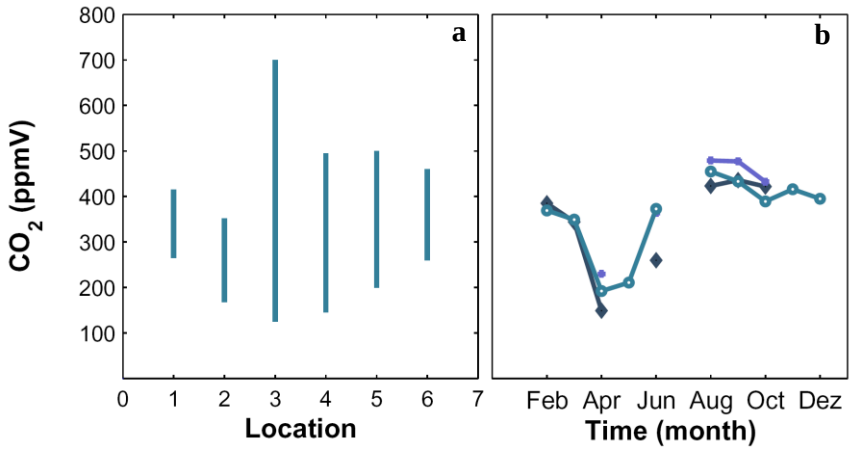

Fig. 7- (a) CO_2 concentration ranges in different locations [*Borges et al.*, 2006]. 1) Galician coast, 2) Barents Sea, 3) Cadiz, 4) North Sea, 5) English Channel, 6) Golf Biscay and Celtic Sea. (b) CO_2 concentrations in the years 2001 (diamonds in dark blue), 2002 (closed circle in navy blue) and 2003 (circle in green-blue) in the Southern Bight of the North Sea [*Schiettecatte et al.*, 2007]. Note that annual variation is higher than inter-annual variation.

Moreover, phytoplankton experiences changes in CO_2 demand as light varies during the day, with cloud cover or while cells are mixed through the water column. When exposed to sudden high light intensity, chlorophyll (Chl) and accessory pigments absorb more energy and increase carbon fixation. In this situation a CCM plays a crucial role in increasing CO_2 concentration close to Rubisco, therefore compensating for increased CO_2 demand. Indeed an increase of the CCM expression has been found when cells were exposed to increasing light intensity [*Beardall*, 1991].

2.7. Effects of increasing CO_2 on marine phytoplankton

Climate influences organisms' diversity and distribution [*Falkowski & Oliver,* 2007]. The expected increase in CO_2 concentrations due to anthropogenic activities might alter phytoplankton communities according to the individual responses of species within a certain community. Members from the various functional groups have key roles in the organic carbon pump (diatoms), in the carbonate counter-pump (coccolithophores) and in the nitrogen cycle (diazotrophs). Changes in the dominant species or even dominant groups could have consequences for the carbon and nitrogen cycles.

In this thesis, ocean acidification effects were analysed in: 1) cyanobacteria, one of the oldest life-forms of Earth and 2) coccolithophores, potentially the most sensitive to ocean acidification. Emphasis was put on bloom forming species, namely the cyanobacteria *Trichodesmium sp*. (marine), *Nodularia spumigena* and *Anabaena sp.* (brackish or freshwater), and the coccolithophore *Emiliania huxleyi.*

2.7.1. Cyanobacteria

The responses of cyanobacteria to projected CO_2 concentrations weren't known until the beginning of this thesis. Since then, several species have been investigated from unicellular non-diazotrophic (*Prochlorococcus* and *Synechococcus,* [*Fu et al.,* 2007]), unicellular diazotrophic (*Crocosphera,* [*Fu et al.,* 2008]), and filamentous non-heterocystous (*Trichodesmium*- [*Barcelos e Ramos et al.,* 2007; *Hutchins et al.,* 2007; *Kranz et al.,* 2009; *Levitan et al.,* 2007]) to filamentous heterocystous (*Nodularia*- *Czerny, Barcelos e Ramos & Riebesell,* 2009; and *Anabaena- Franz et al.,* in prep.).

The CO_2 effect found in cyanobacteria seems to vary with their physiological and morphological specificities. The non-diazotrophic *Prochlorococcus* and *Synechococcus,* two genetically related but differently distributed species [*Olson et al.,* 1990], were hardly affected by increasing CO_2 concentrations (375 and 750ppm) alone [*Fu et al.,* 2007]. In contrast the unicellular diazotroph *Crocosphaera* increased carbon and nitrogen fixation under rising CO_2 concentrations [*Fu et al.,* 2008].

The filamentous diazotrophs' sensitivities to ocean acidification are considered in more detail in the publication, co-authored manuscripts and synthesis, especially the non-heterocystous *Trichodesmium* and the heterocystous *Nodularia spumigena*. Both species are colony-forming cyanobacteria and form extensive blooms. In Baltic Sea blooms

Nodularia spumigena appears associated with *Anabaena sp.*, *Aphanizomenon flos-aquae* and the non-diazothroph *Synechococcus sp.* [*Stal et al.*, 2003].

2.7.2. Coccolithophores

Coccolithophores were one of the first phytoplankton groups to get attention from the ocean acidification community due to the potential effect on calcification. Species considered until now have shown different sensitivities to the CO_2 concentrations projected for the year 2100 [*IPCC*, 2007]. The strongest response to increasing CO_2 concentrations was found in *Gephyrocapsa oceanica*, seen both in the decrease in calcification and increase in organic carbon fixation [*Riebesell et al.*, 2000b]. The majority of the studies done to date with the closely related *Emiliania huxleyi* also found a decrease in calcification rate and a slight increase in photosynthesis in response to elevated CO_2 concentrations under nutrient and light replete conditions [*Feng et al.*, 2008; *Riebesell et al.*, 2000b; *Zondervan et al.*, 2001]. A recent study with several strains of *Emiliania huxleyi* found strain specific sensitivities [*Langer et al.*, 2009]. However, the variability found was small considering that the strains were isolated from very diverse geographical environments (optimal temperature, nutrients, etc.). Besides the laboratory work done with *Emiliania huxleyi* [*Feng et al.*, 2008; *Leonardos & Geider*, 2005; *Riebesell et al.*, 2000a; *Sciandra et al.*, 2003; *Zondervan et al.*, 2001] similar trends were also found in natural communities dominated by this species [*Delille et al.*, 2005; *Riebesell et al.*, 2000b]. However, other species have shown distinct sensitivities within a similar CO_2 range. Indeed *Calcidiscus leptoporus* had an optimum calcification curve and *Coccolithus pelagicus* was not affected by changes in the CO_2 concentration between 150 and 920 µatm [*Langer et al.*, 2006].

2.8. Outline

In this thesis, the publications, manuscripts and co-authored manuscripts report on the effects of global change on phytoplankton, both by varying CO_2 concentrations and light intensity.

In publication I, the response of the single most significant nitrogen fixing cyanobacterium, *Trichodesmium*, to rising CO_2 is investigated. Cell division rate as well as nitrogen and carbon fixation rates increased with rising CO_2 concentrations from 140

to 850 µatm. A possible explanation for the increase of both carbon and nitrogen fixation is discussed to be related to reduced costs for CCM operation. Potential consequences for biogeochemical element cycles and feedbacks to the climate system are also evaluated.

In manuscript II, the short-term response of *Emiliania huxleyi* to abrupt changes in the seawater carbonate system is addressed. Organic and inorganic carbon fixation rates show, within hours, a similar response as previous studies with acclimated cultures. This indicates that *Emiliania huxleyi's* physiology can be quickly adjusted to the response of acclimated cultures. Consequences to the interpretation of community studies are briefly discussed.

In manuscript III, the short-term response of *Emiliania huxleyi* and *Phaeodactylum tricornutum* to a light intensity increase from 50 to 800 µmol m^{-2} s^{-1} is investigated. There are differences between the two species in terms of magnitude and timing of their responses. In fact *Phaeodactylum tricornutum* has a faster increase in diadinoxanthin quota, a slower decrease of the F_v/F_m and a stronger increase in organic carbon fixation rate in the first 10 minutes, while *Emiliania huxleyi* quickly increases inorganic carbon fixation rate (calcification rate). These data are discussed in view of differences in CO_2 demand. Finally, the potential effects on species competitive fitness and distribution are also considered.

Additionally, one manuscript (co-authored manuscript IV) and one manuscript in preparation (co-authored manuscript V) focus on heterocystous cyanobacteria and address whether the response found in *Trichodesmium* can be generalized to other filamentous cyanobacteria. The heterocystous species, contrary to *Trichodesmium*, showed a slight decrease in nitrogen fixation rates and no increase in cellular division rates.

2.9. References

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3. Publications

3.1. List of publications

The work developed for the doctoral thesis resulted in the following publications and manuscripts:

I. J. Barcelos e Ramos, H. Biswas, K. G. Schulz, J. LaRoche and U. Riebesell: Effect of rising atmospheric carbon dioxide on the marine nitrogen fixer *Trichodesmium*, published in *Global Biogeochemical Cycles*, 21, GB2028, doi:10.1029/2006GB002898 (2007).

II. J. Barcelos e Ramos, M. N. Müller and U. Riebesell: Short-term response of the coccolithophore *Emiliania huxleyi* to abrupt changes in seawater carbon dioxide concentrations, *Biogeosciences Discussions*, 6, 4739-4763 (2009).

III. J. Barcelos e Ramos, K. G. Schulz, S. Febiri and U. Riebesell: Photoacclimation to abrupt changes in light intensity of *Phaeodactylum tricornutum* and *Emiliania huxleyi*: light harvesting, dissipation and utilization, to be submitted to *Plant Physiology*.

List of co-authorships

IV. J. Czerny, J. Barcelos e Ramos and U. Riebesell: Influence of elevated CO_2 concentrations on cell division and nitrogen fixation rates in the bloom forming cyanobacterium *Nodularia spumigena*, *Biogeosciences Discussions*, 6, 4279-4304 (2009).

V. J. Franz, J. Barcelos e Ramos and U. Riebesell: Impact of CO_2 on the filamentous Baltic Sea cyanobacterium *Anabaena spec.*, in preparation.

3.2. Contributions to each publication

I. This publication came in the sequence of inconclusive results from Haimanti Biswas. The experimental design was optimized by me and discussed with Kai G. Schulz and Ulf Riebesell. The manuscript was written by me, with comments and scientific support from the co-authors.

II. The concept and experimental design were developed together with Marius N. Müller, and further discussed with Ulf Riebesell. The experiment was carried out by Marius N. Müller and myself, with the help of Peter Wiebe. The paper was written by me, with comments and scientific support from the co-authors.

III. The idea for these experiments was mine, and was discussed with Kai G. Schulz. Experiments were performed with the help of Kai G. Schulz and Sarah Febiri. The paper was written by me, with comments from the co-authors.

Contribution to each co-authored manuscripts

IV. The experimental design for this paper was developed by Jan Czerny in close collaboration with me and further discussed with Ulf Riebesell. Jan Czerny wrote the paper, with comments from the co-authors.

V. The experimental design for this paper was developed by Jasmin Franz in close collaboration with me and further discussed with Ulf Riebesell. The manuscript is being written by Jasmin Franz.

I Effect of rising atmospheric carbon dioxide on the marine nitrogen fixer *Trichodesmium*

J. Barcelos e Ramos, H. Biswas, K. G. Schulz, J. LaRoche, and U. Riebesell

Global Biogeochemical Cycles 21: GB2028

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Effect of rising atmospheric carbon dioxide on the marine nitrogen fixer *Trichodesmium*

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[1] Diazotrophic (N_2-fixing) cyanobacteria provide the biological source of new nitrogen for large parts of the ocean. However, little is known about their sensitivity to global change. Here we show that the single most important nitrogen fixer in today's ocean, *Trichodesmium*, is strongly affected by changes in CO_2 concentrations. Cell division rate doubled with rising CO_2 (glacial to projected year 2100 levels) prompting lower carbon, nitrogen and phosphorus cellular contents, and reduced cell dimensions. N_2 fixation rates per unit of phosphorus utilization as well as C:P and N:P ratios more than doubled at high CO_2, with no change in C:N ratios. This could enhance the productivity of N-limited oligotrophic oceans, drive some of these areas into P limitation, and increase biological carbon sequestration in the ocean. The observed CO_2 sensitivity of *Trichodesmium* could thereby provide a strong negative feedback to atmospheric CO_2 increase.

Citation: Barcelos e Ramos, J., H. Biswas, K. G. Schulz, J. LaRoche, and U. Riebesell (2007), Effect of rising atmospheric carbon dioxide on the marine nitrogen fixer *Trichodesmium*, *Global Biogeochem. Cycles*, *21*, GB2028, doi:10.1029/2006GB002898.

1. Introduction

[2] Diazotrophic cyanobacteria play an important role in marine ecosystems and biogeochemical cycles [*Mulholland*, 2006]. They provide the biological source of new nitrogen in large parts of the oligotrophic ocean [*Codispoti et al.*, 2001]. *Trichodesmium*, a colony-forming cyanobacterium, fixes nitrogen in an area corresponding to almost half of Earth's surface [*Davis and McGillicuddy*, 2006] and is estimated to account for more than half of the new production in parts of the oligotrophic tropical and subtropical oceans [*Capone et al.*, 2005; *Mahaffey et al.*, 2005]. Future expansion of the oligotrophic subtropical provinces to higher latitudes due to surface ocean warming and increased stratification is expected to change the spatial extent of *Trichodesmium* and hence the magnitude of global N_2 fixation by this organism [*Boyd and Doney*, 2002; *Breitbarth et al.*, 2007].

[3] In addition to sea surface warming, the oceans are experiencing another change of global significance related to CO_2 increase, namely the acidification of seawater due to the massive uptake of fossil fuel CO_2 [*Sabine et al.*, 2004]. The corresponding change in seawater carbonate chemistry results in an increase in hydrogen ion activity and CO_2 concentration, along with a corresponding decrease in carbonate ion concentration and carbonate saturation state. Oceanic CO_2 uptake has already caused a reduction in upper ocean pH by 0.1 units [*Feely et al.*, 2004] and is expected to cause a further decrease by 0.3–0.4 units by the end of this century if CO_2 emissions continue at current trends. While effects of CO_2-related seawater acidification have been demonstrated for a variety of marine microalgae and cyanobacteria [*Giordano et al.*, 2005], mainly focusing on carbon acquisition and concentrating mechanisms [*Burkhardt et al.*, 2001], little is known about it's impact on marine diazotrophs [*Levitan et al.*, 2007]. To examine the influence of CO_2–induced changes in seawater chemistry on *Trichodesmium*, we have grown this species over a range of CO_2 concentrations under controlled laboratory conditions.

2. Material and Methods

2.1. Experimental Setup

[4] Semicontinuous batch cultures of *Trichodesmium* IMS101 were kept in exponential growth at CO_2 partial pressures ranging levels from 140 to 850 μatm. This corresponds to pHt (pH on the total scale) values from 8.5 to 7.8 and, for that reason, a sixfold increase in CO_2, a 1.2-fold increase in bicarbonate (HCO_3^-) and a fourfold decrease in CO_3^{2-} concentrations. Cultures were grown in 0.2 μm sterile filtered YBCII medium [*Chen et al.*, 1996] with 5 μmol kg^{-1} phosphate and no nitrate addition, at 25 °C, a photon flux density of 150 μmol m^{-2} s^{-1} (supplied from cool white fluorescent bulbs, Philips TLD 36W/54) and a 14/10 hour light/dark cycle. CO_2 levels were adjusted by different additions of NaOH to media with equal amount of dissolved inorganic carbon (DIC). Cells were acclimatized to the experimental conditions for approximately two months. In the course of each experiment, cultures were allowed to grow in 1 L polycarbonate bottles for a maximum of three generations, so that DIC would not decrease more than 3%. Cultures were always diluted to the same starting chlorophyll *a* (Chl *a*) concentration (approximately 3 μg/L) in order to

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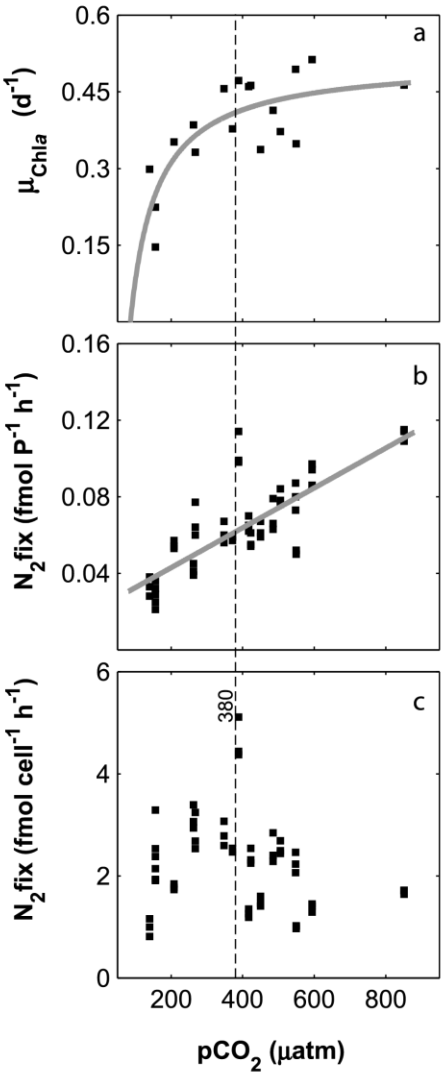

Figure 1. (a) Growth rate based on Chl *a* (μ) of *Trichodesmium* in relation to CO_2 levels (pCO_2). The solid line was obtained by fitting the data to a modified Michaelis-Menten curve which allows for a minimum cellular requirement of CO_2. Rates of nitrogen fixation (N_2fix) (b) per unit of POP and (c) per cell by *Trichodesmium* in relation to CO_2 levels (pCO_2). The solid line in Figure 1b was obtained by linearly fitting the data. In Figures 1b and 1c, triplicate measurements (squares) were made on each sampling day (3 days in total). The vertical dashed line denotes present-day CO_2.

maintain exponential growth. Sampling occurred 4 or 5 days after the previous dilution, when Chl *a* reached approximately 20 μg/L, at a fixed time (10 am) to avoid introducing bias due to diel variability. Cell abundances were determined by cell counts of Lugol preserved samples (2−3% final concentration).

2.2. Carbonate system

[5] CO_2 concentrations were calculated from dissolved inorganic carbon (DIC) and total alkalinity using the temperature- and salinity-dependent dissociation constants given by the *Department of Energy* [1994]. DIC was measured photochemically [*Stoll et al.*, 2001] using an automated segmented-flow analyzer (Quaatro) equipped with an autosampler. Alkalinity was measured according to *Dickson et al.* [2003] in duplicate through potentiometric titration, using a 794 Basic Titrino (Metrohm).

2.3. Nitrogen Fixation

[6] Nitrogen fixation rates were determined by the acetylene reduction assay [*Capone*, 1993], using a gas chromatograph with flame ionization detector (SHIMADZU GC-14B) and calculated according to *Capone* [1993], considering the Bunsen gas solubility coefficient determined for 25°C by *Breitbarth et al.* [2004].

2.4. Cell Dimensions and Numbers

[7] Fresh samples for cell measurements were collected onto GTBP black filters (0.2 μm) under low vacuum (200 mbar). For each condition, fresh preparations were made with immersion oil (IMMERSION OIL-UVFL, SI, Olympus) and cell dimensions were determined (approximately 100 cells measured) using autofluorescence conditions at 1000x magnification (Zeiss optic microscope with a fluorescence lamp). Cell count samples were preserved with Lugol (2−3% final concentration), filtered on polycarbonate filters (25 mm diameter and 0.2 μm pore size) and photographed systematically (80x magnification) along a transect covering the diameter of the filter with additional supplemental photos (Leica MZ12 Binocular) taken randomly (not in the statistical sense). Individual filaments were enumerated and measured with the computer program Image J. Trichome length was then divided by the cell lengths determined for each condition, providing the corresponding number of cells for each sampling day.

2.5. Cell Contents

[8] Chlorophyll *a* samples were analyzed fluorometrically (10-AU Fluorometer, GAT) according to *Derenbach* [1969] and *Welschmeyer* [1994]. Samples for cellular particulate organic carbon (POC) and nitrogen (PON) were analyzed in a gas chromatograph (EURO EA Elemental Analyser, EUROVECTOR equipped with a thermal conductivity detector and an element analyzer) following *Sharp* [1975]. Particulate organic phosphorus (POP) filters were submitted to alkaline persulphate oxidation (adapted from *Hansen and Koroleff* [1999]) and measured colorimetrically by means of a spectrophotometer (UV-1202, UV-VIS Spectrophotometer, SHIMADZU). Cell morphology was observed by inverted microscopy (Zeiss) at 1000x magnification.

3. Results

[9] Over the experimental CO_2 range (140 to 850 μatm), cell division rate of *Trichodesmium* increased about twofold when based on Chl *a* (Figure 1a), POC, PON, POP and cell numbers (data not shown). Nitrogen fixation rate normal-

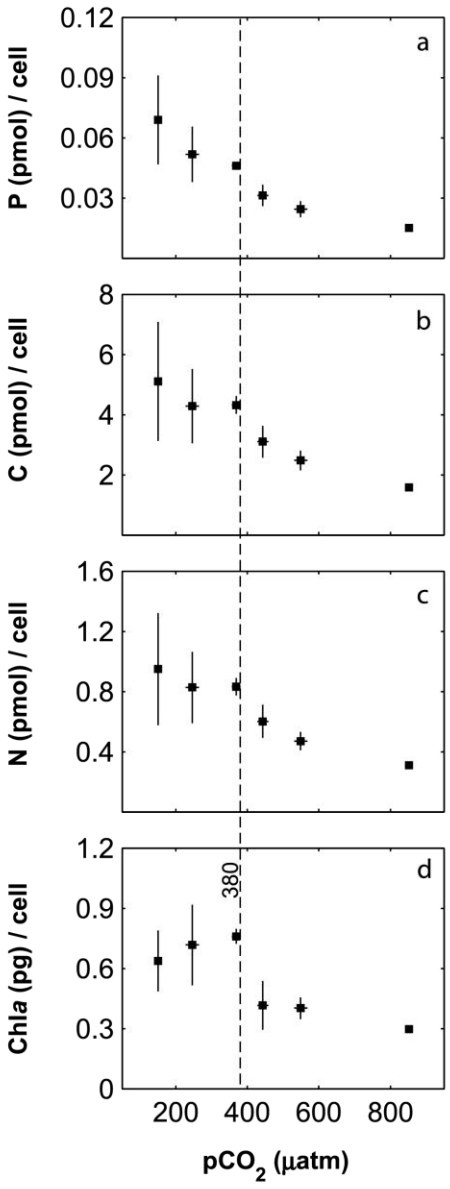

Figure 2. Cellular elemental contents of *Trichodesmium* in relation to CO_2 levels (pCO_2). Cellular contents presented are (a) phosphorus, (b) carbon, (c) nitrogen, and (d) chlorophyll *a*. The squares are averages of defined pCO_2 groups (100 μatm) with vertical and horizontal errors bars representing standard errors for each group. Dashed vertical line denotes present-day CO_2.

ized to cellular phosphorus quota (Figure 1b) and Chl *a* content (data not shown) increased threefold over the experimental CO_2 range. This corresponds to a 50% increase in P-normalized N_2 fixation for atmospheric CO_2 increasing from its present value (380 μatm) to that projected for 2100 (750 μatm) assuming a business as usual CO_2 emission scenario. Owing to the strong reduction in cell content with increasing CO_2 (see below), C and N_2 fixation rate demonstrate no trend with CO_2 when normalized on a per cell basis (Figure 1c).

[10] Increased cell division rate at elevated CO_2 was associated with lower cellular content of C, N, P, and Chl *a* (Figure 2). On average, cell length in high CO_2 grown cultures was ca. 20% lower than under low CO_2 conditions, with no significant difference in cell width. This can be

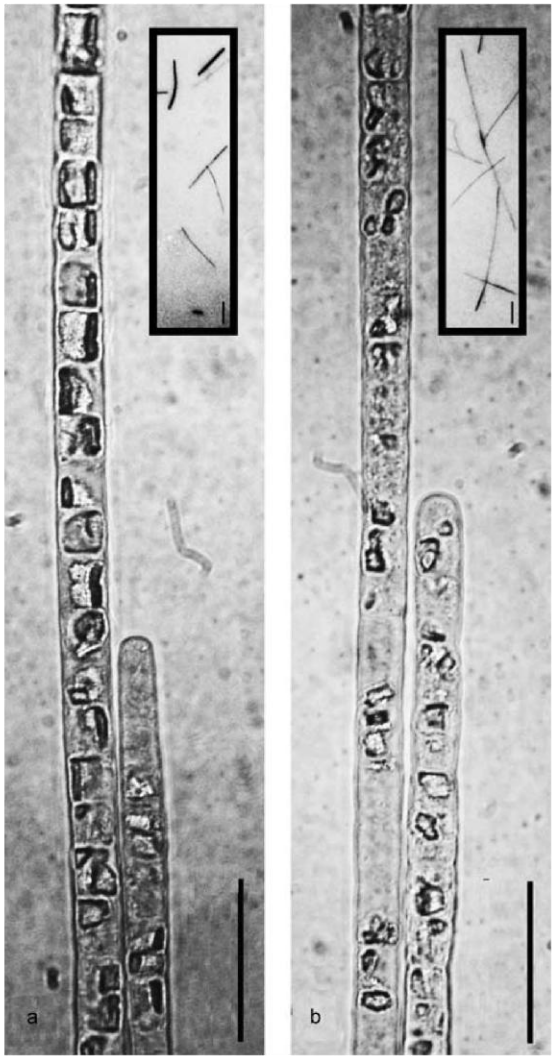

Figure 3. Cell morphology of *Trichodesmium* IMS101 visualized at 1000x magnification acclimatized to (a) 167 μatm and (b) 700 μatm CO_2 levels. Insert: corresponding trichome view at 80x magnification. Scale bars: 25 μm (Figures 3a and 3b), 200 μm (bottom right of inserts).

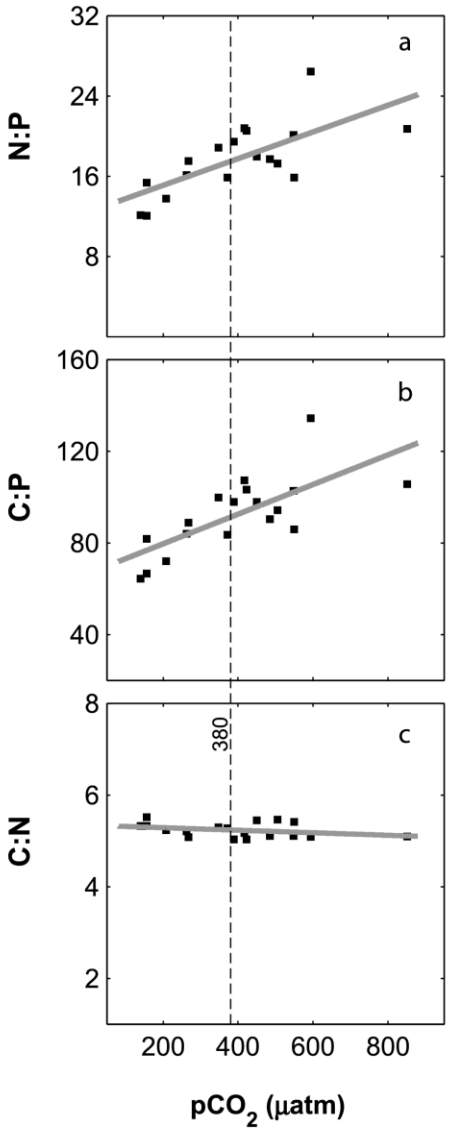

Figure 4. Particulate organic matter ratios of *Trichodesmium* in relation to CO_2 levels (pCO_2). (a) Nitrogen to phosphorus ratio. (b) Carbon to phosphorus ratio. (c) Carbon to nitrogen ratio. The solid lines were obtained by a linear fit through the respective data. The vertical line denotes present-day CO_2.

explained by the larger fraction of newly divided cells in the faster growing high CO_2 cultures. There was also a concomitant 7–35% increase in trichome length (Figure 3 inserts) caused by a higher number of cells per trichome. Consistent with the reduction of cell quotas, cells grown at higher CO_2 appeared less granulated (Figure 3).

[11] CO_2-stimulated C and N_2 fixation in relation to P assimilation caused a distinct shift in cellular stoichiometry. Over the experimental range, N:P and C:P ratios increased from approximately 13:1 and 69:1, respectively, at the low CO_2 to 24:1 and 124:1, respectively, at high CO_2 (Figures 4a and 4b). For the projected increase in atmospheric CO_2 over the course of the 21st century (380 to 750 μatm), this corresponds to an approximately 40–50% increase in N:P and C:P ratios, i.e., consistent with the independently determined change in P normalized N_2 fixation rates. In contrast, the C:N ratio remained unaffected at about 5.2 over the entire CO_2 range (Figure 4c).

4. Discussion

[12] CO_2 concentration has been previously reported to affect photosynthetic carbon fixation of marine phytoplankton [*Hein and Sand-Jensen*, 1997; *Leonardos and Geider*, 2005; *Raven*, 2003; *Riebesell et al.*, 1993], but its significance in modifying oceanic primary production remains uncertain [*Riebesell*, 2004]. More importantly, the magnitude of the observed CO_2 effect on *Trichodesmium* by far exceeds those previously seen in other photoautotrophs. A twofold to threefold increase in N_2 fixation rate per unit of phosphorus utilization and a doubling in growth rate as CO_2 increases from glacial (180 μatm) to year 2100 CO_2 levels (750 μatm) makes *Trichodesmium* one of the most CO_2 sensitive primary producer tested to date [*Giordano et al.*, 2005].

[13] An unusually strong response also occurred with respect to changes in the cellular elemental composition, with C:P and N:P ratios increasing approximately twofold over the experimental CO_2 range (Figure 4). Unlike previous studies [*Burkhardt et al.*, 1999; *Gervais and Riebesell*, 2001], our results for *Trichodesmium* do not indicate a CO_2 saturation level in the range tested, above which elemental ratios remain constant. Moreover, contrary to other phytoplankton groups, which generally increase cellular carbon quotas with increasing CO_2 availability [*Riebesell*, 2004; *Rost et al.*, 2002], C quota (as well as cellular N, P, and Chl *a* contents) of *Trichodesmium* decreases with increasing CO_2. This difference is due to the strong CO_2 effect on cell division rate in *Trichodesmium*, which again is generally not found in other phytoplankton groups to the same extent [*Rost et al.*, 2002].

[14] The nature of the observed CO_2 effect on carbon and nitrogen fixation in *Trichodesmium* is presently unknown. Both photosynthetic carbon fixation and diazotrophic nitrogen fixation are energy demanding processes, which compete for energy and reducing power with a variety of other cellular processes, such as protein synthesis [*Geider and MacIntyre*, 2001] and carbon acquisition [*Kaplan and Reinhold*, 1999]. Owing to the relatively low affinity of their main carboxylating enzyme Rubisco (ribulose bisphosphate carboxylase oxygenase) [*Tortell*, 2000], cyanobacteria have to invest significant amounts of energy to concentrate CO_2 at the site of carboxylation. This so-called CO_2 concentrating mechanism (CCM) includes the cost of active HCO_3^- and CO_2 transport into the cell and to the site of carboxylation, and the synthesis of the proteins involved

in CCM activity (e.g., transporters and carbonic anhydrase). Cyanobacteria are known to down-regulate their CCM in response to increasing CO_2 availability [*Giordano et al.*, 2005], thereby allowing allocation of energy to other cellular processes.

[15] *Trichodesmium* has a low-affinity Na^+/HCO_3^- symport [*Badger et al.*, 2006], which is likely to be dependent on an energy demanding Na^+/H^+ antiport system [*Badger et al.*, 2006; *Giordano et al.*, 2005]. Moreover, this species also has a low-affinity CO_2 uptake complex dependent on reductive power present in the thylakoid membrane [*Badger et al.*, 2006]. Two processes possibly affected by ambient CO_2 concentration are diffusive uptake of CO_2 by the cell and CO_2 leakage out of the cell. Elevated CO_2 concentrations could reduce the diffusive loss of CO_2 from the cell and/or increase the proportion of diffusive CO_2 uptake into the cell, resulting in a down-regulation of CCM activity. As a consequence, competing processes such as C and N_2 fixation may benefit by receiving additional energy and reductive power. In fact, the surprisingly high CO_2 sensitivities of C and N_2 fixation imply major changes in energy allocation in response to changing CO_2 availability. A possible explanation may be found in the low CO_2 affinity of cyanobacterial Rubisco. As one of the oldest life forms on planet Earth [*Falkowski et al.*, 2004] cyanobacteria rely on a Rubisco with lower affinity to CO_2 in relation to O_2 when compared to more recently evolved phytoplanktonic groups [*Tortell*, 2000]. To compensate for this, cyanobacteria need to invest considerable amount of energy in concentrating CO_2 at the site of carboxylation. Thus the energetic gain at elevated CO_2 may be higher in cyanobacteria compared to other phytoplanktonic groups with Rubiscos characterized by higher CO_2 affinities. Clearly, further studies are needed to examine CCM regulation of this ecologically and biogeochemically important diazotroph.

[16] If representative of the natural environment, the observed CO_2 sensitivity of *Trichodesmium* would have broad implications in a changing ocean. The increase of N:P and C:P in *Trichodesmium*-dominated oceanic regimes may affect the nutritional value of primary produced organic matter as well as the stoichiometry of particulate matter sinking to depth. On the ecosystem level this could impact the efficiency of bacterial degradation and zooplankton reproduction, with possible cascading effects on the pelagic food web [*Sterner and Elser*, 2002]. From a biogeochemical point of view, changing C:P and C:N ratios may affect the remineralization depth, the pool of bioavailable nitrogen and consequently carbon sequestration via the biological carbon pump. Extrapolation of this finding to the natural environment should be done with caution, however, since other factors (e.g., iron and phosphorus availability and temperature) may influence *Trichodesmium*'s response to rising CO_2.

[17] Global *Trichodesmium* N_2 fixation, is estimated from direct measurements at 60 to 85 Tg N a^{-1} [*Galloway et al.*, 2004; *LaRoche and Breitbarth*, 2005; *Mahaffey et al.*, 2005], accounting for half of the new production in parts of the tropical and subtropical oceans [*Capone et al.*, 2005]. On the basis of our results, this could rise to 90 to 128 Tg N a^{-1} by 2100 in a business-as-usual CO_2 emission scenario (IS92a) [*Intergovernmental Panel on Climate Change*, 2001]. Moreover, recent estimates of *Trichodesmium* subsurface abundances raise the basin-scale average nitrogen fixation rate by a factor of 2.7 to 5.0 [*Davis and McGillicuddy*, 2006].

[18] In view of recent studies, suggesting that global N_2 fixation by unicellular cyanobacteria may be as high or higher than that of *Trichodesmium* [*Montoya et al.*, 2004; *Zehr et al.*, 2001], it appears important to examine the extent to which the observed CO_2 sensitivity of *Trichodesmium* N_2 fixation also applies to other diazotrophic cyanobacteria.

[19] A CO_2-induced increase in N_2 fixation would increase bioavailable nitrogen in N-limited oligotrophic oceans, either by release of ammonia and dissolved organic nitrogen [*Mulholland et al.*, 2004] or by cell death (programmed cell death, grazing and/or viral attack), possibly fueling productivity of other phytoplanktonic groups. This may enhance phosphate utilization, driving some of these areas to P limitation under future high CO_2 conditions. However, given that other environmental changes are expected with rising CO_2 such as temperature and atmospheric dust deposition, the relative importance of the CO_2 effect on nitrogen fixation needs to be further assessed in the context of the future ocean. The expected rise in global sea surface temperature, leading to enhanced stratification, decreased mixed layer depth and decreased nutrient availability, has been suggested to result in an increase in nitrogen fixation [*Boyd and Doney*, 2002]. Because the increase in temperature may also result in a reduction of the area characterized by optimum nitrogen fixation and growth [*Breitbarth et al.*, 2007], the overall effect of ocean warming on diazotrophs is still uncertain.

[20] We here show that not only *Trichodesmium* responds to rising CO_2, but as one of the oldest life forms on planet Earth it is more sensitive then other groups previously considered (e. g., coccolithophores and diatoms). If the observed effect on *Trichodesmium* is a general phenomenon in diazotrophic cyanobacteria our results would predict an increase in global ocean N_2 fixation at CO_2 levels expected for the future ocean. This in turn, would increase the nitrogen inventory, resulting in increase future primary productivity and oceanic carbon sequestration.

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II Short-term response of the coccolithophore *Emiliania huxleyi* to abrupt changes in seawater carbon dioxide concentrations

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Short-term response of the coccolithophore *Emiliania huxleyi* to abrupt changes in seawater carbon dioxide concentrations

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Abstract

The response of the coccolithophore *Emiliania huxleyi* to rising CO_2 concentrations is well documented in acclimated cultures where cells are exposed to the CO_2 treatments for several generations prior to the experiment. Extended acclimation times have generally been applied because of the lack of information about time required to reach a new physiological "equilibrium" (acclimation) in response to CO_2-induced changes in seawater carbonate chemistry. Here we show that *Emiliania huxleyi*'s short-term response (hours to 1 day) to increasing CO_2 is similar to that obtained with acclimated cultures under comparable conditions in earlier studies. At CO_2 concentrations ranging from glacial (190 μatm) to projected year 2100 (750 μatm) levels, calcification decreased and organic carbon fixation increased within 8 h after exposing the cultures to the changed CO_2 conditions. This led to a decrease in the ratio of $CaCO_3$ to organic carbon production. Our results show that *Emiliania huxleyi* rapidly alters the rates of various essential processes in response to changes in seawater carbonate chemistry, establishing a new physiological (acclimation) "state" within a matter of hours. If this relatively rapid response applies to other phytoplankton species, it may simplify interpretation of studies with natural communities (e.g. mesocosm studies and ship-board incubations), where often it is not feasible to allow for a pre-conditioning phase before starting experimental incubations.

1 Introduction

Until the year 2100 atmospheric CO_2 concentration is expected, for a "business-as-usual" CO_2 emission scenario, to almost triple from pre-industrial values (IPCC, 2007), with a concomitant 45% decrease of CO_3^{2-} ion concentrations and a drop of 0.4 pH units in the surface ocean. Substantial effort has been undertaken to understand phytoplankton responses to these changes, with different laboratory approaches including incubations with dilute (Burkhardt et al., 1999; Riebesell et al., 2000a; Rost et al.,

2003) and dense monoclonal batch cultures (Iglesias-Rodriguez et al., 2008), semi-continuous (Barcelos e Ramos et al., 2007; Fu et al., 2007; Xia and Gao, 2003) and chemostat (Sciandra et al., 2003) cultures, as well as ship-board incubations (Tortell et al., 2002; 2008) and mesocosm field experiments of natural populations (Delille et al., 2005; Engel et al., 2005; Riebesell et al., 2007).

Particular attention has been given to coccolithophores, a group of calcifying marine phytoplankton which was found to exhibit distinct sensitivity to ocean acidification. Members of this group, which is considered responsible for a significant fraction of the pelagic biogenic carbonate precipitation (Milliman, 1993), responded to CO_2 induced seawater acidification by changing cellular calcification rates. The best studied and probably most productive coccolithophore, *Emiliania huxleyi*, has generally been found to decrease its calcification rate in response to elevated CO_2 concentrations under nutrient and light replete conditions (Feng et al., 2008; Riebesell et al., 2000b; Zondervan et al., 2001).

All laboratory work on CO_2/pH sensitivity of *Emiliania huxleyi* so far have used cultures pre-exposed (acclimated) to the experimental CO_2 treatment. While a common acclimation period applied in these studies corresponds to about 9 to 12 generations (Riebesell et al., 2000b; Zondervan et al., 2002; Feng et al., 2008), the actual time needed for acclimation to elevated CO_2 is unknown. Acclimation period refers to the time necessary for individual cells to establish a new physiological "state" in response to a change in the environmental condition.

In cases where an individual's phenotypic plasticity (acclimation) and the population's genotypic variability are insufficient to maintain competitive fitness under changing environmental conditions, a species' survival may depend on its ability to adapt (Bell and Collins, 2008). Projecting a species' long-term response to environmental change therefore requires knowledge about both its acclimation and adaptation potential. Phenotypic plasticity responses to a changing environment may delay, favour or even speed up adaptive evolution (Ghalambor et al., 2007), further complicating attempts of predicting pathways of species evolution and ecosystem development un-

der changing environmental conditions. With regard to *Emiliania huxleyi* it is unknown what role the observed phenotypic plasticity will have in its response to the future CO_2 concentrations, neither if its natural populations will have the potential to adapt to the high CO_2 ocean. It is known that this species has high genetic variability, as reported for *Emiliania huxleyi* blooms (Medlin et al., 1996), but no evolutionary study with this species has been performed to date.

Studies with natural communities are a valuable approach to address questions related to species interactions in response to climate change. Indeed, diatom community's shifts in response to elevated CO_2 concentrations were described in phytoplankton assemblages from the Equatorial Pacific (Tortell et al., 2002) and Southern Ocean (Tortell et al., 2008). In recent mesocosm experiments the most pronounced CO_2 related effect was rather on inorganic carbon uptake and organic carbon loss from the upper water column (Schulz et al., 2008). These types of experiments are often conducted without prior acclimation of the enclosed communities to the CO_2 treatments. The time needed for phytoplankton physiology to respond to abrupt and drastic changes in seawater carbonate chemistry and to what extent this involves a temporary stress response are presently unknown. Considering the importance of studying the potential effects of rising CO_2 on natural communities (e.g. in mesocosm and ship-board incubations) and the relatively limited incubation time in these studies, a better understanding of the relevant time-scales in physiological processes of acclimation is urgently needed.

Thus, in this study *Emiliania huxleyi's* response to an abrupt change in CO_2 concentrations was followed during 26 h and the results were compared to those obtained for acclimated cultures in earlier studies. Furthermore, by following short-term cellular responses we investigate the acclimation time necessary for phytoplankton suddenly exposed to elevated CO_2.

2 Material and methods

2.1 Experimental setup

Monospecific cultures of the coccolithophore *Emiliania huxleyi* (strain isolated during 2005 mesocosm experiment in Bergen by M. N. Müller) were grown at a constant CO_2 concentration (approximately 495 μatm, with a corresponding pH_{total} value of 7.7) for a total of about 20 generations (3 consecutive semi-continuous batch cultures). These pre-cultures were continuously aerated with 0.2 μm filtered ambient (room) air (Rena Air50 aquarium pump), which allowed to grow the cultures to the cell abundance needed as inocula to start the experiment (2.1×10^6 cell ml^{-1}), without major shifts in the CO_2 level. However, while aeration replenishes dissolved inorganic carbon (DIC), calcification reduces total alkalinity (TA), resulting in a decrease in pH and carbonate saturation state (minimum of about 7.7 pH and 0.9 Omega, with a corresponding 495 μatm CO_2) at constant pCO_2. Thus, the carbonate system from both the last pre-culture and the experiment were monitored through TA and DIC measurements. Both pre-cultures and experimental cultures were grown in 0.2 μm sterile filtered North Sea water, at 15°C, a photon flux density of 150 μmol m^{-2} s^{-1} (supplied from cool white fluorescent bulbs, Philips TLD 36 W/54) and a 14/10 h light/dark cycle. Nutrient enrichment followed *f*/2 (Guillard, 1975; Guillard and Ryther, 1962) for the pre-cultures and *f*/20 (88 μmol l^{-1} nitrate and 3.6 μmol l^{-1} phosphate) for the experiment. The carbonate system of the media was adjusted shortly before the day of the experiment by addition of 1 molar NaOH or HCl, simulating well CO_2-induced changes in seawater carbonate chemistry (Schulz et al., 2009). For the experiment, cells were inoculated just before the beginning of the light phase to a starting concentration of about 3.5×10^4 cells ml^{-1} in each of the 4 CO_2 treatments ranging from minimum approximately 182 to maximum 1591 μatm. This corresponded to pH_{total} values ranging from 8.36 to 7.47 with a concomitant 8.5-fold increase in CO_2, a 1.1-fold increase in bicarbonate (HCO_3^-), a 7-fold decrease in carbonate (CO_3^{2-}) concentrations and a calcite saturation state rang-

ing from 7.6 to 1.1 (Table 1). The cell abundance chosen assures that less than 2% DIC was taken up by the cells during the experiment. After carefully mixing the culture inocula with the manipulated media, each CO_2 treatment was subdivided into smaller bottles for the determination of carbon fixation rate, carbonate chemistry, cell numbers and diameter and F_v/F_m. Additionally, samples were taken for scanning electron microscopy. Sampling occurred 2 h, 4 h, 8 h, 14 h, 24 h and 26 h after the start of the first light phase.

2.2 Carbonate system

CO_2 concentrations were calculated from temperature, salinity, phosphate, DIC and TA concentrations using CO2sys (Lewis and Wallace, 1998), with the equilibrium constants given in Roy et al. (1993). DIC was measured photochemically (Stoll et al., 2001) using an automated segmented-flow analyzer (Quaatro) equipped with an autosampler (+/−10 μmol kg^{-1} accuracy and 5 μmol kg^{-1} precision). DIC measurements were calibrated with certified reference material (Dickson standard). Alkalinity was measured according to Dickson et al. (2003) in duplicate (minimum) through potentiometric titration, using a Metrohm Titrando 808 with about 24 μmol kg^{-1} accuracy (calibration with Dickson standard) and 3.5 μmol kg^{-1} precision.

2.3 Carbon fixation

For each data point (time after onset of light: 2 h; 4 h; 8 h; 14 h; and 26 h) 6×65 ml culture flaks were spiked with 100 μl of a 1.85×10^{12} Bq H^{14} CO_3^- solution, of which 4 flasks were incubated under experimental conditions and 2 were kept in the dark. Radioactive label was added to the samples just before the light phases of both experiment days. Duplicate subsamples for total (25 ml) and organic (40 ml) particulate carbon, plus the corresponding darks were filtered onto cellulose acetate (0.45 μm) filters under low pressure (200 mbar). After filtration, 1 ml HCl (0.1 molar) was added to the particulate organic carbon filter (organic carbon fixation) for 30 s, assuring the

dissolution of all calcium carbonate (see Müller et al., 2008). Both filters were rinsed with 0.2 μm filtrated seawater to remove the excess of radioactive dissolved inorganic carbon. Lumagel Plus (Universal LSC cocktail) was then added to the filters in scintillation vials and the radioactivity measured in a Liquid Scintillation Analyser (Tri-Carb 2900TR, Packard). Particulate inorganic carbon fixation (calcification) was calculated as the difference between total carbon (not acidified filters) and organic carbon (acidified filters) fixation.

3 Cell diameter and numbers

Cell abundance and diameter were determined immediately after sampling at each time point by using a Coulter Counter Z series (Beckmann Coulter). Cell division rate (μ) was calculated according to:

$$\mu = (\ln \mathrm{Ce} - \ln \mathrm{Ci})/\Delta t \quad (1)$$

where Ce and Ci refer to end and initial cell concentrations, respectively and Δt to the duration of the experiment in days.

3.1 Maximum photochemical quantum yield of photosystem II (F_v/F_m)

Photosynthetic efficiency was determined as F_v/F_m by using a PAM (PhytoPAM, Phyto-ED Walz, PPAA0138) after a 20 min dark incubation.

3.2 Scanning electron microscopy (SEM)

SEM samples were fixed with formaldehyde (1% final concentration) at each time point. Samples were then filtrated onto polycarbonate filters (0.45 μm pore size) under low pressure (<200 mbar), dried for 12 h at 60°C and glued on aluminium stabs. The filters

were coated with gold-palladium and photographs of the most representative specimens taken with a CamScan-CS-44 (Scanning electron microscope) at the Institute of Geosciences of the Christian Albrechts University in Kiel.

4 Results

After 8 h of exposure to the experimental CO_2 levels (~190 to 1500 μatm) cumulative organic carbon fixation in *Emiliania huxleyi* showed an increasing trend with CO_2 concentration (Fig. 1a). The opposite trend, a decrease with increasing CO_2 was obtained for cellular calcification (Fig. 1b). Due to a stronger decrease in calcification compared to the increase in organic carbon fixation the cumulative total carbon fixation decreased with rising CO_2 (Fig. 1c). Carbon fixation rates were also determined for each period between 2 consecutive sampling points. From 4 to 8 h after the inoculation, organic carbon fixation rate increased 35% from the lowest to the highest CO_2 level (Fig. 2a). For the same period of time, this corresponded to a 19% decrease in the calcification rate from 190 μatm to approximately 800 μatm and 44% from 190 to 1500 μtam (Fig. 2b). Total carbon fixation rates increased during the whole light phase. After 26 h, at the beginning of the new light phase, carbon fixation rates were again at the low levels measured at the start of the experiment (Fig. 2). At this point, organic carbon fixation rates slightly increased and calcification rates slightly decreased from 190 to 1500 μtam.

The ratio of calcification to organic carbon fixation (Calcification/OC_{fix}) decrease with rising CO_2 (Fig. 1d) became evident about 8 h after the inoculation. This trend is maintained even after the start of the next light phase, even though with a smaller slope and absolute values. Scanning electron microscopy after 8 h and 26 h reveals some under-calcified coccoliths on cells exposed to high CO_2 concentration (Fig. 3). The under-calcified coccoliths are mostly in the layer closest to the cells surface, as is expected for newly produced coccoliths. After 8 h the under-calcified coccoliths of the 1500 μtam CO_2 treatment were mostly observed in smaller cells, because it is on those

that the most recently formed layer becomes visible. The 800 μtam CO_2 treatment showed only slight under-calcification both 8 and 26 h after the manipulation.

Cell division rate decreased from 1.01 at ~190 μtam to 0.90 at ~1500 μtam (Fig. 4). Cell size increased during the light phase, with a weak trend of decreasing cell diameters with increasing CO_2 concentrations at the end of the light phase (Fig. 5a). This trend was reversed after cell division. F_v/F_m increased during the light phase and was lower at low CO_2, maintaining the same trend at the beginning of the following light phase (Fig. 5b).

5 Discussion

5.1 From short-term to acclimated response

The effect of increasing CO_2 concentrations in the ocean has generally been assessed by the physiological response of acclimated phytoplankton cultures (from days to weeks). However, virtually nothing is known about their short (within 24 h) and long-term (months to years) response.

5.1.1 Calcification

Our results showed that within hours after the high CO_2 exposure the calcification response of non-acclimated *Emiliania huxleyi* is similar to that observed in acclimated cultures (Riebesell et al., 2000b), under the same light irradiance (150 μmol m^{-2} s^{-1}), temperature (15°C), similar CO_2 range (~190 to 800 μatm) and L/D cycle (in this study 14/10 while other 16/8). In fact, after 8 h we found a 19% decrease in calcification with rising CO_2 concentrations which compares well with the 15.7% found by Riebesell et al. (2000b). In terms of the absolute values, calcification was slightly higher in this compared to the previous study.

Remarkably the decrease in calcification could be seen with scanning electron microscopy already after short-term exposure to high CO_2. Cells grown under elevated

CO_2 levels showed increased numbers of incomplete or under-calcified coccoliths. However, because newly formed coccoliths are positioned at the cell surface and were therefore hidden by a second layer of coccoliths formed under pre-experimental conditions (approx. 1 coccolith per hour, Paasche, 2002), a systematic analysis of the degree of calcification and the frequency of malformations was not possible in this short-term incubation.

5.1.2 Organic carbon fixation and F_v/F_m

As previously reported, elevated CO_2 stimulated organic carbon fixation, although the effect was almost 3-fold higher than observed in an earlier study (Riebesell et al., 2000b). In agreement with the increase of organic carbon fixation rates under enhanced CO_2 conditions there was an increase of the maximum photochemical quantum yield of photosystem II (F_v/F_m) during the first 14 h. After cell division, cells exposed to the "present" CO_2 condition seemed to have the highest F_v/F_m. Interestingly, in cells subjected to a decrease in the CO_2 concentration F_v/F_m decrease within a short period of time and did not recover in the next 26 h. F_v/F_m is lower when the electrons can not be transported as fast as their production. In this case, a decrease in organic carbon fixation rate due to a change in CO_2 supply might be faster then the re-organization of the Calvin-Benson Cycle substrates, with consequent "clogging" of the electron transport chain.

5.1.3 Calcification/OC_{fix}

There was a decrease in the Calcification to OC_{fix} ratio (already 8 h after manipulation) like in previous studies with acclimated cultures within a similar CO_2 range (Riebesell et al., 2000b; Zondervan et al., 2001). However, there was an overall higher Calcification/OC_{fix} which can be explained by higher calcification rates in this study, since the organic carbon fixation was quite similar to that in a previous study (Riebesell et al., 2000b). Interestingly, the decrease of the Calcification/OC_{fix} ratio after the start

of the next light phase had a less pronounced slope with rising CO_2.

5.1.4 Diel cycle

The diurnal variation of cellular calcification and organic carbon fixation was higher than the differences encountered between the CO_2 levels ranging from ~190 to 1500 μatm. This highlights the importance of the timing of sampling during experiments. For most of the time considered, both our study and Zondervan et al. (2002) show higher organic carbon fixation and lower calcification at enhanced CO_2 concentrations. Unlike Zondervan et al. (2002), however, Calcification/OC_{fix} did not continuously decrease during the light phase, but increased in all CO_2 treatments towards the end of the light phase. An explanation for the disparity in results might be that here an extra sampling point was taken closer to the dark phase.

5.2 Cell division rate and diameter

While cell division of *Emilinia huxleyi* was not found to be affected by elevated CO_2 concentrations in previous studies (Buitenhuis et al., 1999; Clark and Flynn, 2000; Rost et al., 2002) a slight decrease in cell division rate with rising CO_2 was observed in this investigation. This difference may be due to the broader range of CO_2 levels applied here. We do not expect the CO_2 effect on cell division rate to be a short-term stress response caused by changing the CO_2 manipulation procedure (aeration in the pre-cultures and non-aeration in the experiment) or other factors derived from the experimental procedure because cell division rate of the 410 μatm treatment (1.01 d^{-1}) was similar to that of the pre-cultures (1.02 $d^{-1} \pm 0.09$, 4 replicates) exposed to similar CO_2 conditions. Moreover, a similar effect on cell division rate was also found during a long-term (>100 generations) high CO_2 exposure by M. N. Müller (personal communication, 2009), indicating that the observed response was unrelated to the abrupt change in CO_2 concentrations applied in this approach. The opposite trend in cell division rate with rising CO_2 concentration has been observed in other phytoplankton groups, such

as diatoms (Riebesell et al., 1993) and the cyanobacterium *Trichodesmium* (Barcelos e Ramos et al., 2007; Hutchins et al., 2007; Levitan et al., 2007). The apparent difference in specific growth rate responses between various taxonomic groups may be related to the process of calcification, but further investigation is needed to clarify this.

In this study, the cell diameter decreased with increasing CO_2 concentration during the first 14 h. This is most likely due to a more pronounced decrease in calcification than the increase of organic carbon fixation with a consequent decrease in the cellular total carbon. After the dark period, when most cells had divided, on average cells exposed to elevated CO_2 levels had a larger cell diameter. This may be due to the slightly lower cell division rate of high CO_2 exposed cells resulting in a larger number of cells which had not yet undergone cell division. Lower cell diameters at the beginning of the experiment in all treatments may have resulted from higher coccolith detachment due to aeration of the pre-culture.

5.3 CO_2 and pH, a combined effect

Rising CO_2 concentration in the ocean also changes pH, $[HCO_3^-]$ and $[CO_3^{2-}]$, so it is hard to separate the potential effect of each parameter individually. Maintaining a high concentration of CO_2 at the site of carboxilation to ensure efficient operation of the CO_2 fixing enzyme ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO) is an energy demanding processe. A CO_2 increase in the surrounding environment of a cell is likely to decrease the net diffusive efflux of CO_2, reducing the energy needed to maintain high CO_2 inside the cell. The lower energetic cost may be used to increase organic carbon fixation. As for calcification, the decrease in the calcite and aragonite saturation states has been connected to the observed decrease in calcification in foraminifera (Bijma et al., 1999) and corals (Langdon et al., 2000; Leclercq and Gattuso, 2002; Leclercq et al., 2000). As coccolithophore calcification occurs intracellularly and there is no evidence of CO_3^{2-}utilization or any known CO_3^{2-} transporters, the observed response may rather reflect sensitivity to a decrease in pH, associated with increased energetic costs of transporting protons generated during calcification

outside the cell.

Based on the observed increase of organic carbon production at high CO_2 concentration one might expect a concomitant increase in cell division rate, but a slight decrease was observed instead. This effect on cell division rate could be a direct consequence of changing seawater pH, affecting cellular acid-base regulation. In a study on 3 red-tide dinoflagellates Hansen et al. (2007) concluded that growth is mostly affected by pH and that inorganic carbon only plays a minor role under low initial dissolved inorganic carbon concentrations and high pH.

Whatever parameter or combination of parameters influences the different cellular rates, here the cells showed a fast physiological adjustment potentially at the expense of intracellular regulation of DIC content and pH. This possibly happened at the regulation level of both transporters in the membrane and electron chain, and/or enzymes.

5.4 Short (acclimated) to long-term experiments, stepping stones in the understanding of the effect of future climate change

Experiments done with acclimated cells often looked at how the individuals of a clonal culture respond to the projected changes in CO_2 concentrations. *Emiliania huxleyi* acclimated (already after hours) to increasing CO_2 concentrations decreased calcification and increased organic carbon fixation rates in several studies (this study; Feng et al., 2008; Riebesell et al., 2000b; Zondervan et al., 2001). While evolutionary adaptation to increasing CO_2 concentrations has so far not been addressed in *Emiliania huxleyi,* helpful information can be obtained from work done with the plant *Arabidopsis thaliana* (Lau et al., 2007), the alga *Chlamydomonas* (Collins and Bell, 2004) and natural populations from CO_2 springs (Collins and Bell, 2006). Both species referred and natural populations from CO_2 springs showed phenotypic changes with increased CO_2 treatments, but no adaptation (e.g. correlations between CO_2 treatment and genetic patterns, heritability). Still, these phenotypic changes might favour or even speed up adaptive evolution. The lack of indications for adaptation reinforces, on the one hand, the importance to further study phenotypic plasticity changes (acclimation) with rising

CO_2 and, on the other hand, to re-evaluate the long-term experimental designs. Some long-term experiments consider that after 1000 generations there is enough genetic variability so that the culture is not clonal anymore and, therefore, can be treated as a population (Collins et al., 2006). Nevertheless, future long-term experiments could allow for more genetic variability by using several clones, preferentially freshly isolated from the same location, and/or inducing sexual reproduction. It is also important to include some CO_2 variability in these experimental setups, since phytoplankton in its natural environment will not evolve under constant CO_2 concentration, but to an average higher concentration with abrupt changes through time. The daily and seasonal changes of CO_2 concentration will be even more pronounced in the future, due to decreasing ocean buffer capacity. Moreover, one has to start considering in both acclimated and long term experiments, that phytoplankton will be exposed to a combined CO_2, temperature and potentially nutrient composition/availability change.

In summary, short/acclimated and long-term experiments provide complementary information about the phytoplankton response to increasing CO_2 or to a combined effect. Ideally, while the short-term approach identifies species phenotypic plasticity, long-term experiments aim to help understanding the adaptation potential to the future ocean.

6 Conclusions

With this work we were able to show that the response of acclimated cultures to rising CO_2 corresponds to establishing a new physiological "equilibrium" through the change of rates of various essential processes, which *Emiliania huxleyi* cells appear to achieve in less than 24 h. This implies that the cellular adjustment to increasing CO_2 concentrations is independent of cell division. If this relatively rapid response applies to other phytoplankton species, it might simplify the interpretation of studies with natural communities (e.g. mesocosm studies and ship-board incubations), where often it is not feasible to allow for a pre-conditioning phase before starting experimental incubations.

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Table 1. Carbonate system data determined from total alkalinity (TA) and dissolved inorganic carbon (DIC) at 15°C, 34 salinity and 3.4 μmol l^{-1} phosphate using CO2sys (Lewis and Wallace, 1998) with the equilibrium constants given in Roy et al. (1993). While all TA values were measured, meaningful values of DIC could only be obtained for the initial water (start) due to storage problems of the remaining samples (calculated DIC in italic). Hence, DIC at 14 h and 26 h were estimated from organic and inorganic carbon fixation (see Fig. 1) and the assumption that respiration during the night and organic carbon fixation in the first 2 h of the following day were cancelling each other. Thus, DIC drawdown after 26 h was corrected for inorganic carbon fixation only from the difference in TA (14 to 26 h).

Sample	Timing	TA (μmol kg^{-1})	DIC (μmol kg^{-1})	pCO_2 (μatm)	pH_{free}	pH_{total}	HCO_3^- (μmol kg^{-1})	CO_3^{2-} (μmol kg^{-1})	CO_2 (μmol kg^{-1})	Omega for calcite
pre-culture	start	2328	2135	502	8.05	7.97	1971	145	19	3.5
pre-culture	end	1084	1029*	495	7.75	7.67	975	36	19	0.9
exp.	0 h	2558	2135	203	8.41	8.33	1822	305	8	7.3
exp.	0 h	2359	2135	432	8.11	8.04	1954	165	16	4.0
exp.	0 h	2228	2135	899	7.81	7.73	2017	85	34	2.0
exp.	0 h	2150	2135	1588	7.56	7.49	2027	48	60	1.2
exp.	14 h	2511	2071	182	8.44	8.36	1750	315	7	7.6
exp.	14 h	2305	2068	386	8.14	8.07	1882	172	15	4.1
exp.	14 h	2183	2069	750	7.87	7.79	1946	95	28	2.3
exp.	14 h	2106	2081	1435	7.60	7.52	1976	51	54	1.2
exp.	26 h	2490	2060	186	8.43	8.35	1746	307	7	7.4
exp.	26 h	2263	2047	418	8.12	8.03	1874	157	16	3.8
exp.	26 h	2174	2065	772	7.86	7.78	1944	92	29	2.2
exp.	26 h	2077	2066	1591	7.55	7.47	1961	45	60	1.1

*Estimated from TA and pH measured through potentiometric titration.

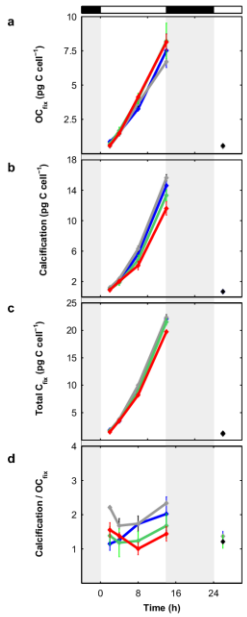

Fig. 1. Cumulative carbon fixation of *Emiliania huxleyi* through time. **(a)** organic carbon fixation per cell, **(b)** calcification per cell and **(c)** total carbon fixation per cell. 190 μatm CO_2 (blue), 410 μatm CO_2 (grey), 800 μatm CO_2 (green), 1500 μatm CO_2 (red). Data from the 26 h considers only a 2 h incubation period. Each CO_2 level has duplicate measurements. Vertical error bars represent the range of the data and the lines connect the averages of each time point. The white/black bar on top represents the light/dark diel cycle, vertical grey bars denote the dark phase.

4759

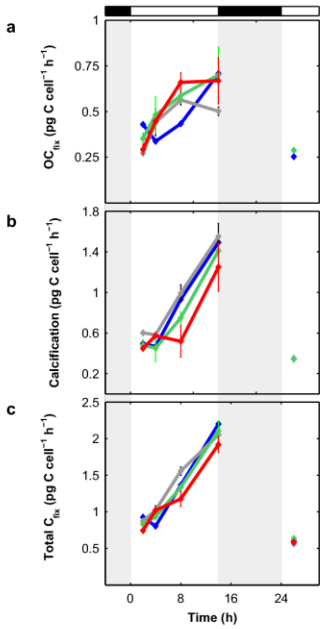

Fig. 2. Carbon fixation rates of *Emiliania huxleyi* determined for each period of time between consecutive sampling points. **(a)** organic carbon fixation per cell per h, **(b)** calcification per cell per h and **(c)** total carbon fixation per cell per h. For each period of time the data point marks the end of the incubation. Each CO_2 level has duplicate measurements. Vertical error bars represent the range of the data and the lines connect the averages of each time point. Line and color coding as in Fig. 1.

4760

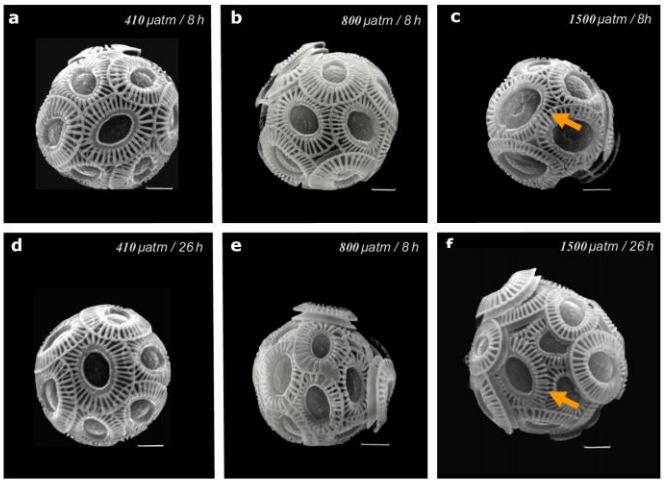

Fig. 3. Scanning electron microscope pictures of *Emiliania huxleyi* grown under different CO_2 concentrations after 8 h of exposure to **(a)** 410 μatm, (**b** and **e**) 800 μatm and **(c)** 1500 μatm and after 26 h of exposure to **(d)** 410 μatm and **(f)** 1500 μatm. The photos chosen are representative of the trend observed. Note the presence of under-calcified coccoliths under enhanced CO_2 conditions, especially visible in the connections between the elements forming the "outer ring" (orange arrows) and in the frequent enlargement of the central area. For the 800 μatm treatment both photographs correspond to cells exposed to the increase on CO_2 concentrations for 8 h, because no differences were found within the time considered (8 and 26 h) and the photographs taken after 26 h were not well focused. Scale bars correspond to 1 μm.

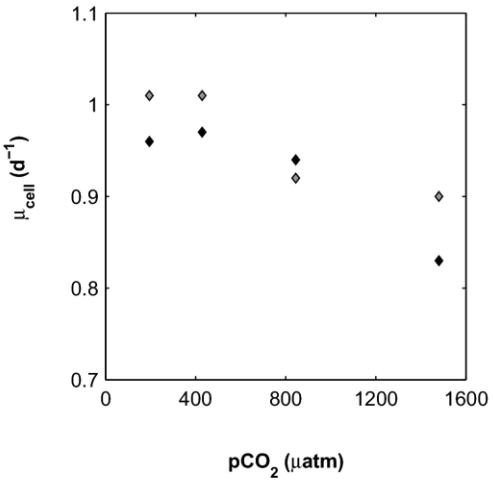

Fig. 4. Cell division rate based on cell counts (μ) of *Emiliania huxleyi* in relation to CO_2 levels (pCO_2). Black diamonds correspond to measurements done at the time of CO_2 manipulation and beginning of the light phase (0 h to 24 h), grey diamonds correspond to measurements done 2 h after that (2 h to 26 h).

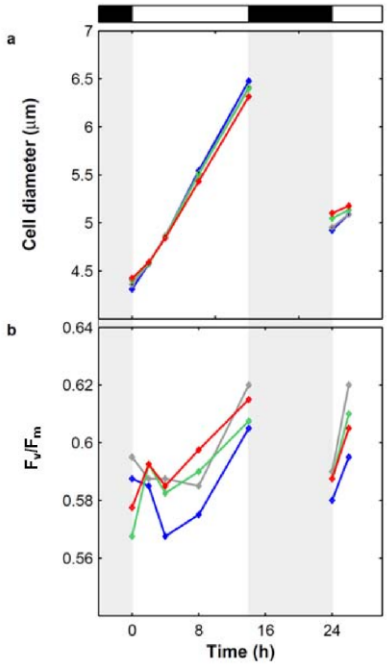

Fig. 5. **(a)** Cell diameter and **(b)** F_v/F_m of *Emiliania huxleyi* through time. In Fig. 6a each CO_2 level has 4 measurements from each bottle with vertical error bars representing standard errors. 190 μatm CO_2 (blue), 410 μatm CO_2 (grey), 800 μatm CO_2 (green), 1500 μatm CO_2 (red). The white/black bar on top represents the light/dark diel cycle, vertical grey bars denote the dark phase.

III Photoacclimation to abrupt changes in light intensity of *Phaeodactylum tricornutum* and *Emiliania huxleyi*: light harvesting, dissipation and utilization

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Abstract

In their natural environment phytoplankton is subjected to abrupt light variation. How cells respond to this, influences their competitiveness in a specific ecological niche. However, photoacclimation to abrupt changes in light intensity and species-specific characteristics are still not fully understood. We show that the estuarine / coastal diatom *Phaeodactylum tricornutum* and the cosmopolitan coccolithophore *Emiliania huxleyi* respond to an abrupt increase in irradiance by 1) decreasing light absorption through the decrease of light harvesting pigments, 2) increasing energy dissipation through the xanthophyll cycle and 3) increasing carbon fixation rates. More importantly we found differences between the species in terms of magnitude and timing of their individual responses. Namely, *Emiliania huxleyi* had a more pronounced decrease in chlorophyll *a* and fucoxanthin cellular contents after the light intensity increase. *Phaeodactylum tricornutum* had a faster increase in diadinoxanthin quota, a slower decrease of F_v / F_m and a stronger increase of organic carbon fixation rate in the first 10 minutes. Strikingly, *Emiliania huxleyi* also increased quickly inorganic carbon fixation rate (calcification rate), and therefore, used this process as an additional sink for excess energy. These findings provide further evidence that there are species-specific responses to the abrupt light changes, potentially even by the functional group (e.g. diatom and coccolithophores). These differences might be translated to competitive relationships between co-existing species and therefore, have consequences at the community level.

Introduction

Photosynthetic organisms require light as energy source for carbon fixation and, therefore, life. As a result of constantly shifting cloud cover they have to adjust to abrupt light variation in their natural environment (Falkowski, 1980). Phytoplankton, unicellular planktonic photoautotrophs, have to deal with additional rapid light fluctuations while passively transported through the water column or when exposed to radiation focusing and defocusing by surface waves (Dera and Stramski, 1986). When brought to the surface, phytoplankton cells experience light intensities orders of magnitude higher than at deeper depths. The increase in light intensity can be so high that light absorption by chlorophyll (Chl) and accessory pigments exceeds the potential utilization. As a consequence, cells have developed mechanisms to dissipate excess energy.

Chlorophyll relaxation from its excited state occurs by the following ways: light emission (fluorescence); reactive oxygen species production; heat dissipation (non photochemical quenching- NPQ); and photochemistry (fueling photosynthesis) (Müller et al., 2001). These energy dissipation valves have specific contributions in photoprotection, for instance chlorophyll fluorescence can account for 0.6 to 3 % of the absorbed photons (Krause and Weis, 1991) and the production of reactive oxygen species for 4 to 25 % (Foyer and Harbinson, 1999).

Reactive oxygen species production may lead to pigment bleaching and death under extreme high light conditions. As concurrent processes, NPQ and photochemistry minimize the production of the damaging O_2 reactive byproducts of photosynthesis.

NPQ is correlated with the xanthophyll cycle (Horton et al., 1996; Bassi and Caffarri, 2000; Müller et al., 2001), which is controlled by the light induced proton gradient across the thylakoid membrane (Lavaud and Kroth, 2006). Under increasing light conditions the xanthophyll cycle of phytoplankton dissipates energy while diadinoxanthin (Dd) is de-epoxidized to diatoxanthin (Dt). If light intensity decreases, Dt is converted to Dd instead (Brunet et al., 2008).

Cells can change the quantity (Fisher et al., 1989) and activity of photosynthetic enzymes, mostly related to carbon fixation (photochemistry), and, therefore, modify their photosynthetic and cell division rates. Indeed, broad light variation influences the ribulose-1,5-biphosphate carboxylase / oxygenase (RuBisCO), by either modulating its cell content (Lin and Carpenter, 1997), its ratio in respect to chlorophyll or protein amount (Fisher et al.,

1989) or its activity. Other factors such as Calvin-Benson substrates also affect photochemistry rates, but are harder to quantify. Even though it is known that part of the excess light can fuel photochemistry there are still uncertainties related to the timing and magnitude of this response.

Light intensity variation increases the demand of carbon available for carbon fixation, eventually beyond the supply. This pressure on carbon supply might have implications for the carbon concentrating mechanisms (CCMs) which increases CO_2 concentrations close to Rubisco. Hence, there might be a dual role for CCMs. On the one hand, they are vital in providing high CO_2 concentration for carbon fixation and adjusting to variable CO_2 supply (Giordano et al., 2005) and, on the other hand, they might play an important role compensating for the changing CO_2 demand in a light variable ocean.

The photoprotective capacity and regulation varies between the phytoplankton groups (Wagner et al., 2006), therefore potentially influencing phytoplankton community composition. Studies with diatoms have correlated their distribution with their photoprotective capacity (Lavaud et al., 2007) as well as with their photosynthetic architecture (particularly, lower concentrations of photosystem I and cytochrome b_6f complex in oceanic diatoms compared to coastal species) (Strzepek and Harrison, 2004). In this context, estuarine / coastal species such as *Phaeodactylum tricornutum* have a higher photoprotection capacity than oceanic diatoms (Lavaud et al., 2007). No study was done to date evaluating competitive fitness under changing light conditions between diatoms and coccolithophores. Nevertheless, coccolithophores, calcifying phytoplankton, might have specificities related to their carbon fixation pathway or due to the additional process of inorganic carbon fixation (calcification).

In summary, understanding phytoplankton's regulation capacity to light intensity variation needs to consider both the cellular contents of photoprotective pigments and carbon fixation rates. Differences in the magnitude and time necessary to channel part of the extra energy into carbon fixation (organic and inorganic) may have important consequences at the community level and species distribution.

To assess the influence of light intensity increase on phytoplankton physiology, we grew a opportunistic representatives of diatoms (*Phaeodactylum tricornutum*) and of coccolithophores (*Emiliania huxleyi*) under controlled laboratory conditions.

Material and Methods

Experimental setup

Monospecific cultures of the coccolithophore *Emiliania huxleyi* (strain PML B92/11A) and the diatom *Phaeodactylum tricornutum* (CCMP632) were grown in modified F/2 media (5 µmol L^{-1} phosphate, 40 µmol L^{-1} nitrate and 40 µmol L^{-1} silicate) at 17 ˚C, a photon flux density of 50 µmol m^{-2} s^{-1} (supplied from Daylight 12-950 lamps, Philips TLD 18W) and a 13.5 / 10.5 h light / dark cycle. A minimum of two consecutive pre-cultures were allowed to grow for 14 generations in total, under low abundance to avoid carbon limitation. Cell numbers in the days of the experiments were about 20 000 to 30 000 cells mL^{-1}. Both the pre-cultures and experiment cultures were mixed daily, providing homogeneous light supply. Each experiment (baseline and light change regimes) was performed two times with duplicate bottles (Figure 1). Therefore, all parameters have 4 data points, with the exception of pigment concentrations which have only 2 (one experiment with duplicate bottles). On the baseline experiment, cells were maintained at the conditions they were acclimated to (50 µmol m^{-2} s^{-1}) in the pre-cultures (Figure 1a). On the experiment day of the light change regime, the light intensity was abruptly raised to 800 µmol m^{-2} s^{-1} 7 hours after the beginning of the light phase (14:00 h) (Figure 1b). Sampling occurred throughout the light phase, starting in the beginning of the light phase (7:00 h, t0) in both the baseline and the light change regime.

Carbon fixation (^{14}C)

Before (2 hours) the dark phase previous to the experiments, cultures (55 ml) were transferred to smaller bottles (65 ml) in order to reduce prolonged handling stress on the day of the experiment. Both light and dark (wrapped in aluminum foil) samples were spiked with 100 µl of a 1.85×10^{12} Bq $H^{14}CO_3^-$ solution and were incubated in the same chamber. Flasks were put in a position that assured equal light intensities to all vials. *Phaeodactylum tricornutum* and *Emiliania huxleyi* samples for particulate organic carbon (plus corresponding darks) were filtered onto cellulose acetate filters (0.45 µm) under low pressure (200 mbar). In the case of *Emiliania huxleyi* an additional filter was made for total carbon and the filters for particulate organic carbon were rinsed with 1 ml HCl (0.01 M) running through, plus 1 ml of HCl (0.01 M) for 30 seconds. The acid addition assured the dissolution of all calcium carbonate (coccoliths). All filters were rinsed with 0.2 µm filtered seawater, removing excess radioactive dissolved inorganic carbon and then placed in scintillation vials. Lumagel Plus

(Universal LSC cocktail) was added to the filters and the radioactive decay was measured in a Liquid Scintillation Analyser (Tri-Carb 2900TR, Packard) after 12 h in the dark. Particulate inorganic carbon fixation (calcification) was calculated as the difference between total carbon (not acidified filters) and organic carbon (acidified filters) fixation.

Cell pigments

Pigment samples were analysed by HPLC (column-Microsorb-MV 100-3C8, 100 x 4.6 mm x ¼, Waters) according to Barlow et al. (1997).

Maximum photochemical quantum yield of photosystem II (F_v/F_m)

Photosynthetic health was determined as F_v/F_m by using a PhytoPAM (Phyto-ED Walz, PPAA0138), after a 20 minutes dark incubation.

Carbonate system

DIC was measured photochemically (Stoll et al., 2001) using an automated segmented-flow analyzer (Quaatro) equipped with an auto-sampler.

Cell diameter and numbers

Cell abundance and diameter were determined shortly after each sampling by using a Coulter Counter Z series (Beckmann Coulter). Cell division rate was calculated as μ according to the expression presented below:

$$\mu=(\ln Ce - \ln Ci) / \Delta t$$

where Ce and Ci refers to end and initial cell concentrations, respectively, and Δt to the duration of the experiment in days. The periods of time considered for the figure 7 were 0 to 10 hours, 0 to 12 hours and 6 to 12 hours after the light phase starts.

Results

After the exposure to a 16 times (1500 %) light intensity increase (50 to 800 μmol m^{-2} s^{-1}), cellular chlorophyll *a* (Chl *a*) (Figure 2a), fucoxanthin (Figure 2b) and chlorophyll *c* (Chl *c*) (Figure 2c) concentrations decrease in comparison to values of cells acclimated to 50 μmol m^{-2} s^{-1} (baseline). Approximately 1 hour after the irradiance increase, Chl *a* cellular contents start

decreasing in relation to the baseline and, at the end of the light phase, are 16.1 % lower in the case of *Phaeodactylum tricornutum* and 22.7 % in *Emiliania huxleyi*. Within 1 hour, cellular concentrations of fucoxantin decrease 5.4 % in *Phaeodactylum tricornutum* and 14.8 % in *Emiliania huxleyi*. At the end of the light phase this decrease is much more pronounced, reaching 23.5 % in *Phaeodactylum tricornutum* and 55.8 % in *Emiliania huxleyi*. Within the same period of time, *Emiliania huxleyi* 19 - Hexatoxanthin quota seems to slightly increase with rising light intensity in comparison to the baseline (data not shown). Chlorophyll *c* decreases, but it is difficult to determine the timing of this response to rising irradiance.

The photoprotective response could also be detected through changes in the xanthophyll cycle. While cellular content of diadinoxanthin increases within 1 hour 39.9 % in *Phaeodactylum tricornutum*, *Emiliania huxleyi* quotas do not change much on this time scale (1.1 % increase). However, when a 3 hours period is integrated, the increase in diadinoxanthin quotas of *Emiliania huxleyi* reach 61.5 % and are even slightly higher than the 57.1 % of *Phaeodactylum tricornutum* (Figure 3a). At the end of the light phase, the increase of diadinoxanthin is 75.7 % in *Phaeodactylum tricornutum* and 76.8 % in *Emiliania huxleyi*. Diatoxanthin increases in both species immediately after the rise of the light intensity. Probably in less then 30 minutes diatoxanthin increases 148.3 % from the baseline values in *Emiliania huxleyi* (Figure 3b). In *Phaeodactylum tricornutum* it is not possible to calculate the percentage of increase since diatoxantin is only detectable in cells exposed to the increase of light intensity (800 µmol m^{-2} s^{-1}).

After the dark period, when most of cell division occurs, cellular concentrations of all pigments considered here decrease from the values of the end of the previous day, in both baseline and in the light change regime. Moreover, most of these pigment concentrations are also lower than the values that cells acclimated to 50 µmol m^{-2} s^{-1} had at the same time of the day. Exceptions are: 1) diatoxanthin cellular content of *Phaeodactylum tricornutum*, which could only be measured after the abrupt increase of the light intensity and can still be measured after the dark period, and 2) the ratio between diadinoxanthin and Chl *a* of *Emiliania huxleyi* which has higher values than the previous day at the same time.

Even though the species considered have different cellular pigment concentrations, the ratio between diadinoxanthin to Chl *a* evolves similarly in both of them (Figure 4a). The sum of all carotenoids (diadinoxanthin, diatoxanthin, fucoxanthin, β-Carotenoid, Chl *c*)

normalized to Chla *a* doesn't change with the light intensity increase in either species, but has higher values for *Emiliania huxleyi* than *Phaeodactylum tricornutum* (Figure 4b).

Immediately after the increase of light intensity, F_v/F_m decreases in both species (8.4 % *Phaeodactylum tricornutum* and 22.6 % in *Emiliania huxleyi* after 10 minutes) and doesn't recover to values found for the cells acclimated to 50 µmol m^{-2} s^{-1} during the rest of the light phase (Figure 5a). While the percentage of decrease in *Emiliania huxleyi* remains about 22.6 % for the next hour, *Phaeodactylum tricornutum* gradually decreases, first to 16.3 % (30 minutes) and then to 22.4 % (1 hour). By the end of the light phase, F_v / F_m of cells exposed to rising irradiance decreases to 19.8 % in *Phaeodactylum tricornutum* and 25.3 % in *Emiliania huxleyi.* After 2 days under high light intensity (800 µmol m^{-2} s^{-1}) *Phaeodactylum tricornutum* F_v / F_m remains higher than *Emiliania huxleyi.* However, after the third day *Phaeodactylum tricornutum* values decrease and *Emiliania huxleyi* slightly increase (Figure 5b).

Part of the excess light is channelled into photochemistry. Both the experiments done for determining the baseline and those with the light increase show increasing carbon fixation, as well as other cellular contents (see above) throughout the light period (Figure 6). Shortly after the light intensity increase organic carbon fixation (mostly photosynthesis considering that other carbon fixation mechanisms may exist) of both *Phaeodactylum tricornutum* and *Emiliania huxleyi* increase above the values found for the baseline (Figure 5a). In fact, after only 10 minutes (14:10 h) there is an approximate 878 % increase of organic carbon fixation rate (calculated from the slopes) for *Phaeodactylum tricornutum* and a 425 % increase for *Emiliania huxleyi* (Figure 6a). If the first 30 minutes are considered instead, changes in organic carbon fixation rates are 377 % and 341 %, respectively. After 1 hour of exposure to the higher light intensity the increase of organic carbon fixation rates in relation to values obtained for the cells acclimated to 50 µmol m^{-2} s^{-1} can not be sustained in either species. If the whole light period after the increase of irradiance is integrated than *Phaeodactylum tricornutum* increases organic carbon fixation rates by 71.2 % while *Emiliania huxleyi* increases 168.4 %. The slower increase of organic carbon fixation rate in the first 10 minutes in *Emiliania huxleyi* is associated with a very quick increase (985 %) of inorganic carbon fixation rate (calcification rate) and therefore a 722 % increase of total carbon fixation rate (Figure 6b).

In the following light phase (still under 800 µmol m^{-2} s^{-1}), organic carbon fixation increases to values higher then those from the previous day, having potentially

Phaeodactylum tricornutum a faster increase after the light is turn on. No evident trend is seen in the inorganic carbon fixation rates.

The cell division rate decreases (Figure 7) from approximately 0.95 (+ / - 0.11) in the baseline to 0.68 (+ / - 0.13) for *Phaeodactylum tricornutum* and 0.59 (+ / - 0.09) to 0.27 (+ / - 0.06) for *Emiliania huxleyi* after the abrupt increase of the light conditions (50 to 800 µmol m^{-2} s^{-1}). If the dark phase is included (period when most of cell division occurs) then both species have similar increase on cell division rates from about 0.85 under 50 µmol m^{-2} s^{-1} to approximately 1.1 after the irradiance increase. The balance between the increase in carbon fixation and the decrease of cell division rates can be seen in a slight increase of the mean cell diameter (Figure 8)

Discussion

Physiological plasticity of unicellular phytoplankton often allows a strong, quick (minutes to days) and reversible response to light intensity change (Falkowski, 1980). This photoacclimation involves several photoprotection mechanisms (Figure 9) which react in parallel, but not necessarily simultaneously. Even though phytoplankton's capacity for photoprotection has been often emphasized, little is known about its timing, species-specific characteristics (e.g. Strzepek & Harrison, 2004) and the amount of excess energy that is in fact used in photochemistry to produce biomass (Wagner et al., 2006). The potential increase in photochemistry with rising light intensity is, most likely, moderated by CCM efficiency of the species, since light intensity variation may occur in a matter of seconds, increasing the demand of carbon available for carbon fixation, eventually beyond the supply. Still little attention has been given to the relation between changes in carbon demand, CCM efficiency and competitive advantage (Rost et al., 2006).

Photoacclimation can be better understood when considering more than one of the photoprotection processes. Here, we followed the acclimation response of *Phaeodactylum tricornutum* and *Emiliania huxleyi* in terms of their light harvesting and dissipation capacity, rate of electron transfer and effective use of energy absorbed (photochemistry). Consequences of these mechanisms for cell division rates and potential implications for their competitive advantages are also discussed.

Light harvesting

Although both species decreased their light harvesting pigment concentrations after the irradiance increase (as in previous works e. g. Fisher et al., 1989), absolute values of Chl *a* and fucoxantin quotas of *Emiliania huxleyi* remained lower during the whole day. This difference may well be size related, since even though the species considered in this work have similar diameter, *Phaeodactylum tricornutum* is superior in volume (length about 18 to 26 µm in this strain, DOE Joint Genome Institute, 2008, about 4 µm diameter in both). In diatoms, the Chl *a* - specific light absorption coefficient has been seen to decrease with increasing cell size (Geider et al., 1986). Thus, the bigger *Phaeodactylum tricornutum* with potentially lower light absorption coefficient may need a higher Chl *a* content than *Emiliania huxleyi.* Additionally, pigment quotas might vary with light intensity, time of the day (pigments accumulate until the end of the light phase when cell division occurs) and species-specific characteristic. Here both species were exposed to the same light intensities and samples were collected simultaneously during the light phase, therefore excluding these possibilities.

Another difference between the two species is the stronger decrease of Chl *a* quota in *Emiliania huxleyi* after the irradiance increase. Although this pronounced response could suggest higher phenotypic plasticity of the coccolithophore, the overall flexibility is determined by the stronger response of *Phaeodactylum tricornutum*'s xanthophyll cycle (see below).

Non photochemical quenching

Changes in light harvesting capacity are important in photoprotection, but it is in energy dissipation that cells invest the most. Indeed, both species investigated here and in previous studies (Lavaud et al., 2003) have a higher percentage of increase in diadinoxanthin and diatoxanthin after rising irradiance than decrease in fucoxanthin, Chl *c* or Chl *a*. Reminding that the xanthophyll cycle consists of an enzymatic controlled conversion of pigments (Brunet et al., 2008) namely diadinoxanthin to diatoxanthin under increasing irradiance, the increase in diatoxanthin found here could suggest the opposite diadinoxanthin trend instead. The reason for this apparent paradox response is that the increase in diadinoxanthin cellular contents is superior to that being converted into diatoxanthin. Diadinoxanthin may be produced from fucoxanthin as it is seen in the fucoxanthin decrease. In fact, Lavaud et al.

(2003) describes the replacement of some subunits rich in fucoxanthin by other rich in diadinoxanthin in diatoms acclimated to intermittent light instead of continuous light. Exposure to several or a single (as tested here) irradiance increase has similar phenotypic expression, for instance the increase on diadinoxanthin has been also reported for *Phaeodactylum tricornutum* under intermittent light (Lavaud et al., 2002).

The increase of the cellular concentration of diadinoxanthin took 3 hours in *Emiliania huxleyi* and only 1 hour in *Phaeodactylum tricornutum* after the light intensity increase. Diatoxanthin, on the other hand, increased practically immediately in both species. The formation of diatoxanthin determines the onset of NPQ within the light-harvesting complexes (Olaizola et al., 1994; Kashino et al., 2002; Lavaud et al., 2002), which reduces the photoinhibitory damage to the antenna of PSII after exposure to rising light as seen in previous work with *Phaeodactylum tricornutum* (Ting and Owens, 1994). Even though cells regulate the xanthophyll cycle on relatively short time scales (Lavaud et al., 2004; Dimier et al., 2007), there hasn't been as much emphasis on their short-term photoacclimation ability. Yet it is on this time scale that differences between species determine advantage in the environment. The slight time lag between the response of *Emiliania huxleyi* and *Phaeodactylum tricornutum* may have major implications in an environment where such changes are frequent and sudden, as coastal or estuarine areas.

Pigment ratios

The proportion of diadinoxanthin to Chl *a* increased with rising light intensity in both *Emiliania huxleyi* and *Phaeodactylum tricornutum*. This change on Chl *a* specific xanthophylls pool affects the potential for dissipating light excess through NPQ (Kashino et al., 2002).

The sum of all carotenoids per Chl *a* did not change with the increase on light intensity in any of the species considered here, which agrees well with the finding that the sum of all carotenoids is genetically predefined while the cellular content of every single carotenoid changes with the light intensity (Leonardos and Harris, 2006).

Emiliania huxleyi had slightly higher absolute values in the ratio between total carotenoids and Chl *a* than *Phaeodactylum tricornutum*. Furthermore, *Emiliania huxleyi* Dd / Chl *a* was prolonged into the next day. That might be related to the origin niche. The oceanic *Emiliania*

huxleyi experiences more constant light conditions than the estuarine *Phaeodactylum tricornutum.*

F_v / F_m

The abrupt increase in irradiance decreased F_v / F_m of both species. What happens is that with the abrupt light intensity increase there is a concomitant increase of absorbed energy to be transported. Its passage through the electron chain depends on the redox state of the proteins performing the electron transport, especially plastoquinone (Falkowski and Chen, 2003). The increase in the electron transfer, but not comparable increase on the velocity of the Calvin-Benson cycle (possibly due to limited substrates) depletes $NADP^+$ and ADP. This causes the "clogging" of the electron chain and is measurable as the decrease of F_v / F_m. *Emiliania huxleyi* had a higher percentage of decrease in F_v / F_m than *Phaeodactylum tricornutum* with consequent lower organic carbon fixation (see below). However, *Emiliania huxleyi* seemed to slightly recover the F_v / F_m values after 3 days under 800 µmol m^{-2} s^{-1}, at the same time that *Phaeodactylum tricornutum* decreased its values. However, the data for the days after have high variation and should be analyzed with care.

Photochemistry

After the light intensity increase, carbon fixation increased dramatically. In fact, organic carbon fixation rate of *Phaeodactylum tricornutum* increased 878 % when the light intensity increased 1500 %. Hence, the cells were able to use a considerable amount of the light increment in the first 10 minutes, probably partly explained by a increase in Rubisco content. It is known that *Phaeodactylum tricornutum* reduces its Rubisco content as well as the maximal rate of light saturated photosynthesis (Pmax) levels when cells are adapted to low light conditions (Beardall and Morris, 1976). However, this type of regulation (Rubisco content) can't fully explain the percentage of increase found in this study.

In *Emiliania huxleyi*, organic carbon fixation rate did not increase as dramatically (444 %) in the first 10 minutes, but the calcification rate (978 %). Therefore, calcification had a very relevant role dissipating the excess energy during the first minutes. This may contradict the suggestion that the ability of acclimating phenotypically to light changes is independent of calcification (Leonardos and Harris, 2006). It is unclear whether the increase of calcification only occurs to dissipate the excess energy or has another benefit for the cell (e.g. could a

higher number of coccoliths per cell refract more light?). It has been seen that cell division rate of calcifying *Emiliania huxleyi* becomes higher than the non-calcifying one under increasing irradiance, even though both calcifying and non-calcifying strains of *Emiliania huxleyi* have a very similar response to variation of the light intensity (e.g. pigment composition) (Leonardos and Harris, 2006). Thus, calcification might be an advantage within coccolithophores inhabiting a light fluctuation environment. This importance in dissipating excess energy and therefore decreasing photoinhibition is also clear in a study where the non-calcifying haploid phase exhibits photoinhibition already above 400 µmol m^{-2} s^{-1} while the calcifying diploid did not show photoinhibition below 1000 µmol m^{-2} s^{-1}(Houdan et al., 2005).

The difference in the magnitude and tempo of organic carbon fixation between the two species may also be the consequence of a lower carbon use capacity (organic) in *Emiliania huxleyi* than *Phaeodactylum tricornutum*, either by lower or less flexible transport capacity into the pyrenoids (lower CCM efficiency), differences in organic carbon fixation (C_4 - like in diatoms) and / or different "stock" concentration of substrates for the Calvin-Benson Cycle. According to previous investigations there are evidences for the dependence of carbon dioxide reduction on the concentration of substrates ready when the light intensity increases (Emerson and Arnold, 1932). Still, one can not exclude the other possibilities as precursors of the species-specific response.

When cells experience an increase in the light intensity, they demand more CO_2 than under the acclimated low light conditions, forcing the CCM to quickly adjust. This adjustment implies spending extra energy (Figure 9). In *Chlamydomonas reinhardtii*, cells that were grown under low CO_2 concentrations used up to 43 % of the ATP formed by photosynthesis in their CCM activity (Yokota et al., 1987). Future work should explore further the role of calcification as light dissipating mechanism and its relationship with CCM efficiency.

The abrupt initial increase of organic carbon fixation is not sustained after 30 minutes in either species, potentially due to: 1) the readjustment of pigments concentrations; 2) the high cost of carbon acquisition; 3) limiting concentrations of Calvin-Benson cycle substrates; or 4) related to Rubisco activity. In Barley leaves the regeneration of the substrate ribulose-1,5-bisphosphate limits CO_2 assimilation and not Rubisco activity (Dujardyn and Foyer, 1989). However, it is hard to determine with certainty the limiting factor in our data.

Shortly after the light intensity rise, total carbon fixed increased stronger in *Phaeodactylum tricornutum* than *Emiliania huxleyi*. This might indicate a more efficient overall CCM which enables *Phaeodactylum tricornutum* to respond more quickly to light fluctuations. Or the difference might lay in the presence of a C_4 mechanism in *Phaeodactylum tricornutum* as found in *Thalassiosira weissflogii* (Reinfelder et al., 2004) and potentially C_3 in *Emiliania huxleyi* (Tsuji et al., 2008).

Cell division rate

Cell division rate decreased hours after the increase in light intensity. Cells acclimated to high irradiance have usually higher cell division rates than those acclimated to lower light conditions. The discrepancy to our results probably results from a momentarily readjustment of the cells to the abrupt change in opposition to already acclimated cells. In fact, when the dark period is considered in the calculation of cell division rate both species have similar values and increased after the rise in light intensity.

Implications for functional groups distribution

Phytoplankton species occurrence and biomass depend on several abiotic conditions such as nutrients / CO_2 availability, mixing, temperature and light intensity. Light intensity in the well mixed surface layer varies between long periods of low irradiance and abrupt high irradiance, reaching even supersaturation (Falkowski & Wirick, 1983). Differences in the capacity to respond to these quick variations, influences the distribution of phytoplankton species or functional groups throughout different niches as those defining estuarine, coastal and oceanic areas. Part of photoprotection varies in its extent and regulation according to the phytoplanktonic group (e.g. Wagner et al., 2006). Diatoms generally dominate in more turbulent coastal areas in opposition to coccolithophores which occur predominantly in oceanic environments. Within diatoms previous studies found differences between the photosynthetic apparatus of offshore and estuarine / coastal species, being *Phaeodactylum tricornutum* the most flexible considered (Lavaud et al., 2007). The explanation seems to be that because oceanic phytoplankton evolved in oceanic iron-poor areas, they have developed lower iron requirements (Sunda et al., 1991; Sunda and Huntsman, 1995). Particularly, this decreased iron demand in oceanic diatoms as *Thalassiosira oceanica* is correlated to lower concentrations of photosystem I and cytochrome b_6f complex (Strzepek and Harrison, 2004),

at the expense of a less efficient short-term energy dissipation through heat (Munekage et al., 2001). While this short term capacity effectively dissipates the excess light it isn't very often needed in a stable environment where irradiance changes occur within hours to days (MacIntyre et al., 2000). In contrast, in a turbulent estuarine or coastal environment phytoplankton cells need to avoid potential damage caused by irradiance variation in a matter of minutes. In fact, estuarine species are able to maintain their cell division rate (Lavaud et al., 2007) and the coastal red alga *Chondrus cripus* even increases growth rate (Greene and Gerard, 1990) under fluctuating light regimes.

The most abundant coccolithophore in the modern ocean is *Emiliania huxleyi* (Paasche, 2002) and one of the most flexible diatoms is the estuarine / coastal *Phaeodactylum tricornutum*. The tempo of activation of changes in species phenotypic plasticity to light variation provides acclimation advantage in their origin environment. *Phaeodactylum tricornutum* showed properties important in a light fluctuating regime (estuarine / coastal), for instance it increased dramatically carbon fixation rate in a short period of time and decreased more gradually its F_v/F_m than *Emiliania huxleyi*. This response can not be sustained. In fact, if a longer period of time was integrated instead than the differences between the two species were not as pronounced, confirming that *Phaeodactylum tricornutum*'s has advantage only on short time scales. The difference between the species considered here can not be fully extrapolated to their functional groups, but might help understanding the general trend.

In the future CO_2 supply is expected to increase (IPCC, 2007) and light availability to change in the surface ocean (due to increased on global average temperature and consequent stratification), if carbon fixation and cell division rates are affected there might have implications to the phytoplankton communities.

Conclusions

We found pigment regulation to occur from less then minutes to 3 hours. This agrees well with the time frame appointed by (Falkowski and LaRoche, 1991) in the definition of photoacclimation, but is shorter than proposed by Fisher et al. (1989).

While *Phaeodactylum tricornutum* cells can utilize most of the excess energy for organic carbon fixation in a short period of time (10 minutes), *Emiliania huxleyi* didn't increase organic carbon fixation rate as fast and dissipates significant portions of the excess energy via calcification rate instead. The higher percentage of increase in organic carbon fixation rate

from *Phaeodactylum tricornutum* than *Emiliania huxleyi* can be explained by a higher flexibility in the CO_2 uptake (CCM efficiency) or storage (C_4 mechanism) and / or initial availability of substrates and enzymes involved in the electron chain and carbon fixation.

In today's ocean, phytoplanktonic groups (such as diatoms and coccolithophores) have different phenotypical plasticity to react to abrupt environmental changes such as light. Therefore, how and how fast species respond may dictate phytoplankton assemblages present in the different niches. In the future, the CO_2 supply is expected to increase and the light availability to change in the surface ocean, its effect on carbon fixation and cell division rates might have implications at the community level.

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Figure legends

Fig. 1. Light intensity through time. (a) baseline- light intensity constant at 50 µmol m^{-2} s^{-1}, (b) light change-light intensity increases abruptly from 50 to 800 µmol m^{-2} s^{-1} 7 hours after the light phase start (14:00 h). The white / black bar on top represents the light / dark diel cycle. The vertical dotted line marks the light intensity increase.

Fig. 2. Cellular pigment content of *Phaeodactylum tricornutum* (Pt) and *Emiliania huxleyi* (Ehux) through time. (a) Chlorophyll *a* (Chl *a*) per cell, (b) Fucoxanthin (Fuco) per cell and (c) Chlorophyll *c* (Chl *c*) per cell. Pt values under the baseline regime (thin black line, black diamonds), Pt values under the light change regime (thick black line, black circles), Ehux values under the baseline regime (thin grey line, grey diamonds) and Ehux values under the light change regime (thick grey line, grey circles). Each light intensity profile has duplicate measurements for each species. Lines correspond to a tendency line based on the average of both data points. The white / black bar on top represents the light / dark diel cycle. The vertical dotted line marks the light intensity increase.

Fig. 3. Pigment cellular content of *Phaeodactylum tricornutum* (Pt) and *Emiliania huxleyi* (Ehux) through time. (a) Diadinoxanthin (Dd) per cell and (b) Diatoxanthin (Dt) per cell. Pt values under the baseline regime (thin black line, black diamonds), Pt values under the light change regime (thick black line, black circles), Ehux values under the baseline regime (thin grey line, grey diamonds) and Ehux values under the light change regime (thick grey line, grey circles). Each light intensity profile has duplicate measurements for each species. Lines correspond to a tendency line based on a average of both data points. The white / black bar on top represents the light / dark diel cycle. The vertical dotted line marks the light intensity increase.

Fig. 4. Pigment ratios of *Phaeodactylum tricornutum* (Pt) and *Emiliania huxleyi* (Ehux) through time. (a) Diadinoxanthin (Dd) per Chlorophyll *a* (Chl *a*) and (b) total carotenoids per Chlorophyll *a* (Chl *a*). Pt values under the baseline regime (thin black line, black diamonds), Pt values under the light change regime (thick black line, black circles), Ehux values under the baseline regime (thin grey line, grey diamonds) and Ehux values under the light change regime (thick grey line, grey circles). Each light intensity profile has duplicate measurements

for each species. Lines correspond to a tendency line based on a average of both data points. The white / black bar on top represents the light / dark diel cycle. The vertical dotted line marks the light intensity increase.

Fig. 5. Maximum photochemical quantum yield of photosystem II (F_v/F_m) of *Phaeodactylum tricornutum* (Pt) and *Emiliania huxleyi* (Ehux) through time. (a) F_v/F_m within 15h and (b) F_v/F_m until 5 days after the light intensity was raised and maintained at 800 µmol m^{-2} s^{-1}. Pt values under the baseline regime (thin black line, black diamonds), Pt values under the light change regime (thick black line, black circles), Ehux values under the baseline regime (thin grey line, grey diamonds) and Ehux values under the light change regime (thick grey line, grey circles). Each light intensity profile has 4 measurements for each species. Lines correspond to the mean and the vertical error bars represent standard deviation of those means at each time point. The white / black bar on top represents the light / dark diel cycle. The vertical dotted line marks the light intensity increase.

Fig. 6. Cumulative carbon fixation of *Phaeodactylum tricornutum* (Pt) and *Emiliania huxleyi* (Ehux) through time. (a) organic carbon fixation per cell of both species (OCfix), (b) carbon fixation per cell of Ehux (Cfix-total, organic and inorganic carbon fixation per cell). Pt values under the baseline regime (thin black line, black diamonds), Pt values under the light change regime (thick black line, black circles), Ehux organic carbon fixation values under the baseline regime (thin light-grey line, light-grey diamonds), Ehux organic carbon fixation values under the light change regime (thick light-grey line, light-grey circles), Ehux inorganic carbon fixation values under the baseline regime (thin dark-grey line, dark-grey diamonds), Ehux inorganic carbon fixation values under the light change regime (thick dark-grey line, dark-grey circles), Ehux total carbon fixation values under the baseline regime (thin medium-grey line, medium-grey diamonds) and Ehux total carbon fixation values under the light change regime (thick medium-grey line, medium-grey circles). Each light intensity profile has 4 measurements for each species. Lines depict mean values and the vertical error bars represent standard errors of those means at each time point. The white / black bar on top represents the light / dark diel cycle. The vertical dotted line marks the light intensity increase. The baseline values from *Phaeodactylum tricornutum* were multiplied by 0.85 for optical reasons, having no implications on the calculations.

Fig. 7. Cell division rate based on cell counts (μ_{cell}) of *Phaeodactylum tricornutum* (Pt) and *Emiliania huxleyi* (Ehux) in relation to the light intensity regimes. Cell division rate was calculated from 3 time periods (0 to 10 hours, 0 to 12 hours and 6 to 12 hours after the light phase starts). Pt data refers to duplicate bottles and Ehux to one bottle. Vertical error bars represent standard deviations of the means of each species and at light condition considered.

Fig. 8. Mean cell diameter through time. (a) *Emiliania huxleyi* and (b) *Phaeodactylum tricornutum*. Pt values under the baseline regime (thin black line, black diamonds), Pt values under the light change regime (thick black line, black circles), Ehux values under the baseline regime (thin grey line, grey diamonds) and Ehux values under the light change regime (thick grey line, grey circles). Each light intensity profile has 4 measurements for each species. Lines depict mean values and the vertical error bars represent standard errors of those means at each time point. The white / black bar on top represents the light / dark diel cycle. The vertical dotted line marks the light intensity increase.

Fig. 9. Schematic diagram of a model eukaryotic cell based on *Phaeodactylum tricornutum* and *Emiliania huxleyi*. Light (arrows coming from the surrounding environment) is absorbed by Chlorophyll *a* (**Chl *a***) exciting it (**Chl a^***). Chlorophyll can relax from its excited state by emitting light (**fluorescence**), fuelling photosynthesis (**photochemistry**), dissipating energy as heat (Non photochemical quenching- **NPQ**) and / or producing, by intersystem crossing, **Chl a^3** which can then produce **O_2^*** (reactive oxygen species). Energy dissipation through photochemistry starts with extracting electrons from water in the photosystem II (**PSII**), transferring them to the cytochrome b_6/f (**cyt bf**) and then to the photosystem I (**PSI**), where **NADP** (nicotinamide adenine dinucleotide phosphate) is reduced to **NADPH** (reduced NADP). The dashed grey line represents the electron-transport chain. The energy and reductive power formed can then be used to fix carbon in the pyrenoide (grey circle), which is a globular structure enriched with Rubisco and encountered in the chloroplast (Dodge, 1973). The chloroplast has 4 membranes in both diatoms and coccolithophores (represented with only 2 lines). Inorganic Carbon acquisition characteristics, as DIC species involved, transporters or transport complexes and energy requirements is still uncertain for both species. Therefore, carbon acquisition to the cell, chloroplast and calcification vesicle

has several question marks. *Phaeodactylum tricornutum* is known to uptake both CO_2 and HCO_3^- species (Matsuda et al., 2001). Words in blue highlight the parameters measured here. Blue arrows indicate the effect found after the light intensity increase. C_3 /C_4 with a question mark stand for the uncertainties associated with carbon fixation in phytoplankton. Some diatoms have been found to operate a C_4 photosynthesis and *Emiliania huxleyi* might execute a C_3 (Tsuji et al., 2008). Abbreviations: ADP (adenosine diphosphate) ATP (adenosine triphosphate). General scheme based on Müller et al. (2001) and Falkowski & Raven (2007).

Figures

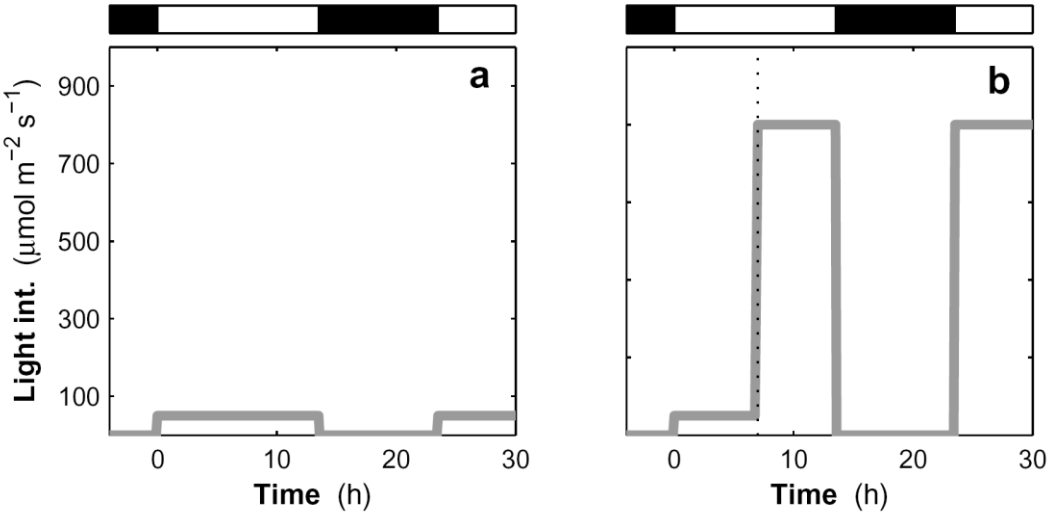
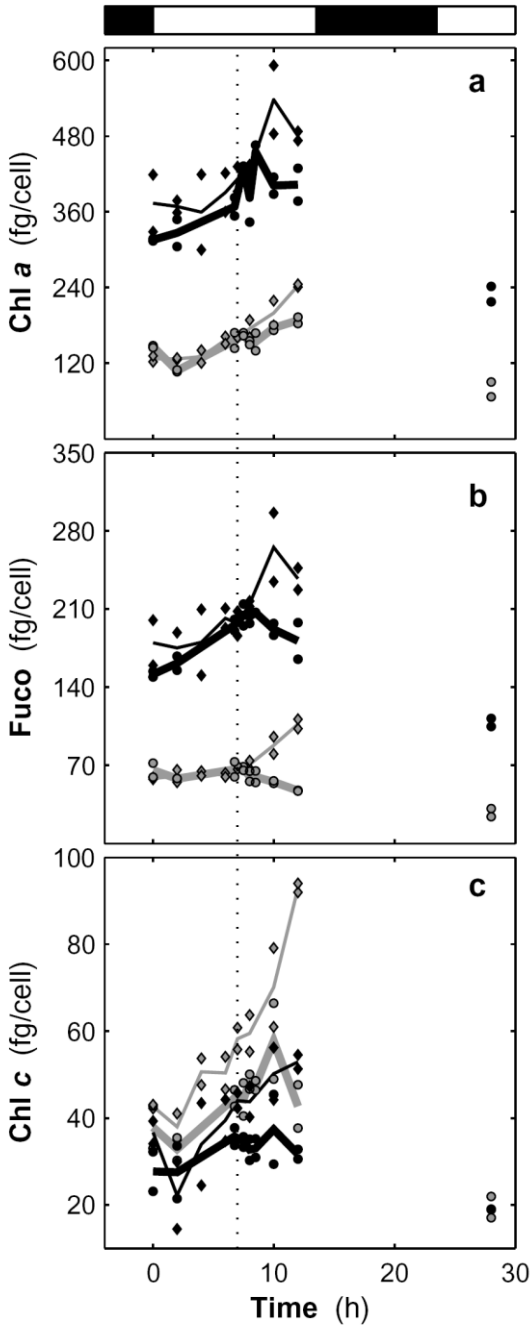


Figure 1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Time (h)

Figure 2

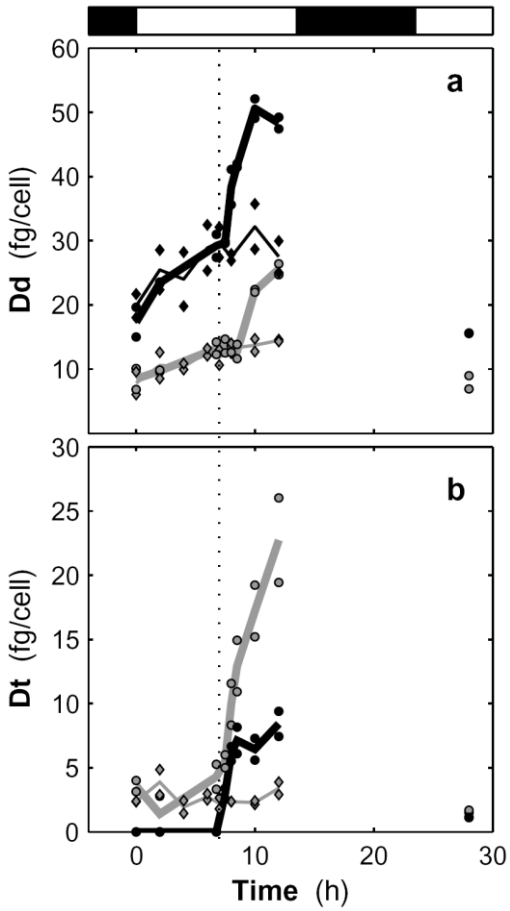
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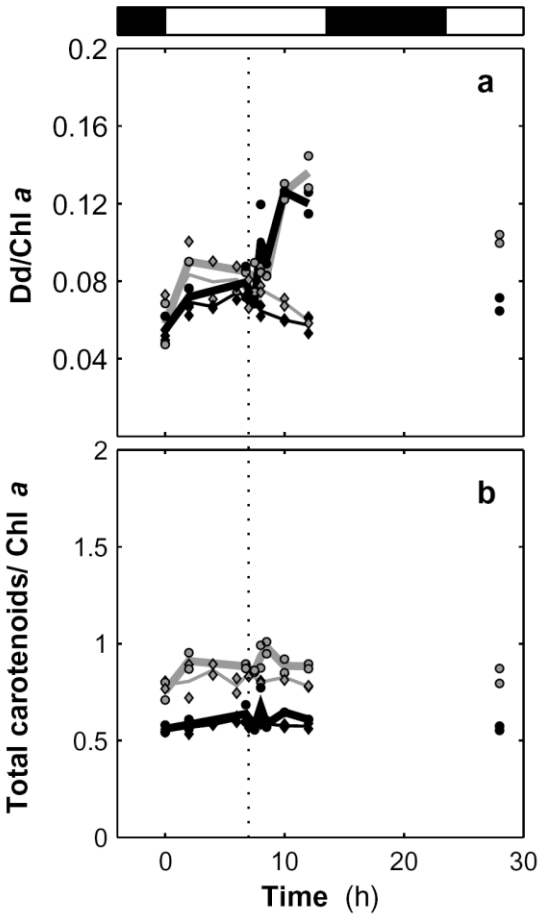
Figure 3



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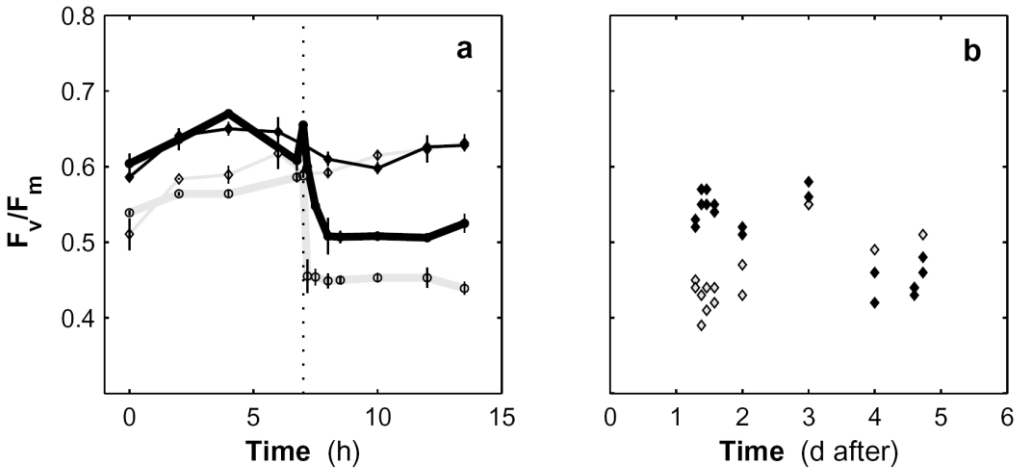

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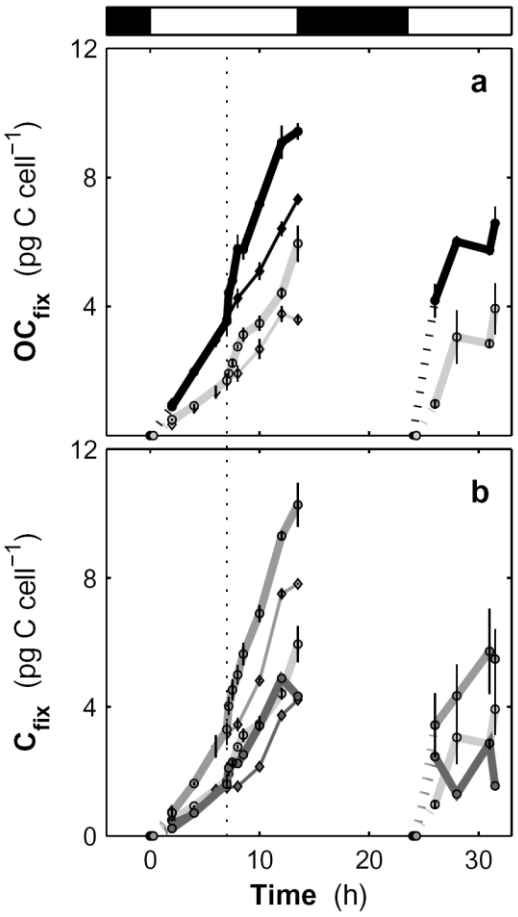

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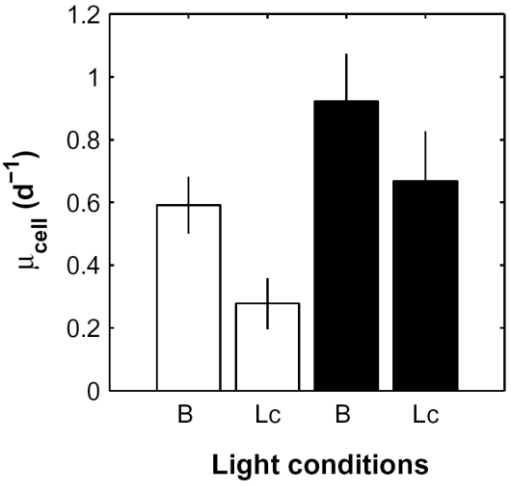

Figure 7

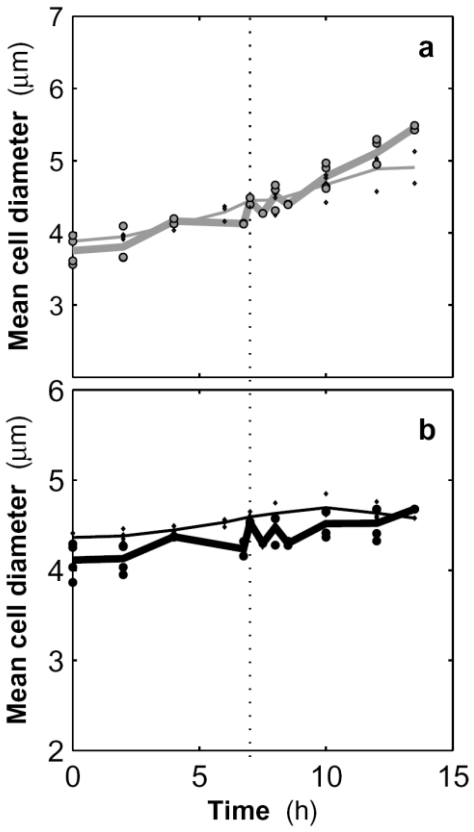

Figure 8

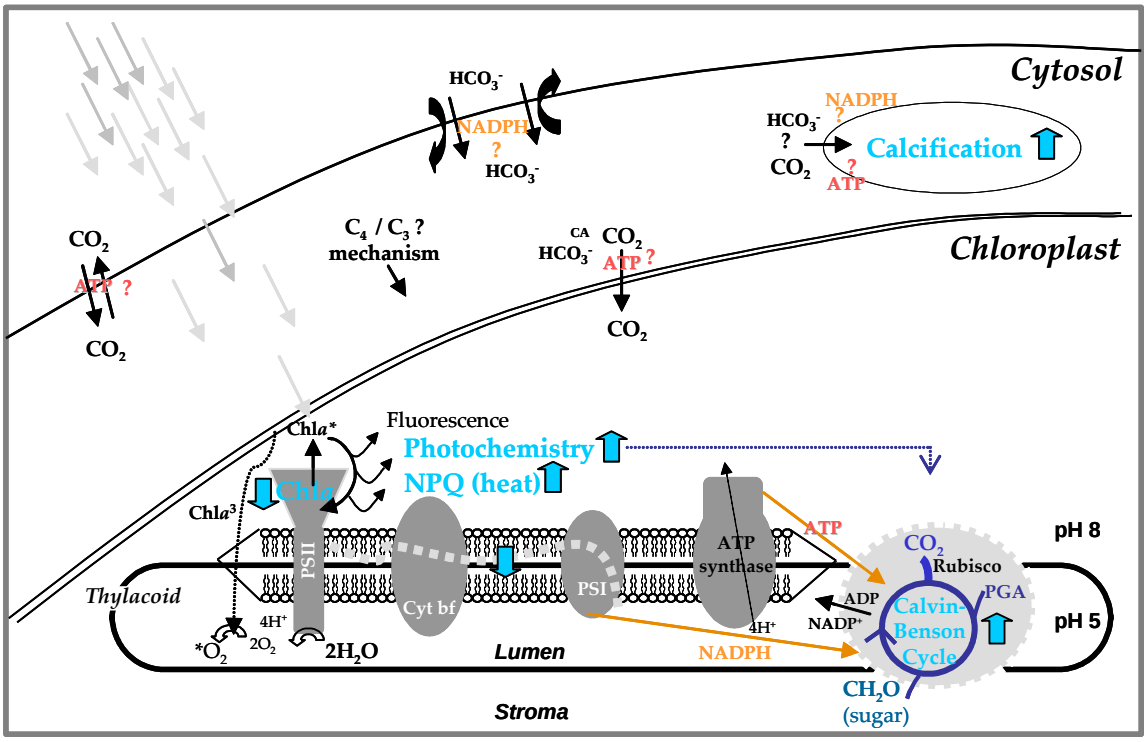

Figure 9

IV Influence of elevated CO_2 concentrations on cell division and nitrogen fixation rates in the bloom-forming cyanobacterium *Nodularia spumigena*

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Influence of elevated CO_2 concentrations on cell division and nitrogen fixation rates in the bloom-forming cyanobacterium *Nodularia spumigena*

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Abstract

The surface ocean currently absorbs about one-fourth of the CO_2 emitted to the atmosphere from human activities. As this CO_2 dissolves in seawater, it reacts with seawater to form carbonic acid, increasing ocean acidity and shifting the partitioning of inorganic carbon species towards increased CO_2 at the expense of CO_3^{2-} concentrations. While the decrease in $[CO_3^{2-}]$ and/or increase in $[H^+]$ has been found to adversely affect many calcifying organisms, some photosynthetic organisms appear to benefit from increasing $[CO_2]$. Among these is the cyanobacterium *Trichodesmium*, a predominant diazotroph (nitrogen-fixing) in large parts of the oligotrophic oceans, which responded with increased carbon and nitrogen fixation at elevated pCO_2. With the mechanism underlying this CO_2 stimulation still unknown, the question arises whether this is a common response of diazotrophic cyanobacteria. In this study we therefore investigate the physiological response of *Nodularia spumigena*, a heterocystous bloom-forming diazotroph of the Baltic Sea, to CO_2-induced changes in seawater carbonate chemistry. *N. spumigena* reacted to seawater acidification/carbonation with reduced cell division rates and nitrogen fixation rates, accompanied by significant changes in carbon and phosphorus quota and elemental composition of the formed biomass. Possible explanations for the contrasting physiological responses of *Nodularia* compared to *Trichodesmium* may be found in the different ecological strategies of non-heterocystous (*Trichodesmium*) and heterocystous (*Nodularia*) cyanobacteria.

1 Introduction

Massive anthropogenic emissions caused atmospheric CO_2 concentrations to rise from an interglacial level of 280 ppm in preindustrial times, (Indermuehle et al., 1999) to presently 380 ppm (Keeling and Whorf, 2005). In the case of unabated CO_2 emissions this value is expected to double until the end of the century (IPCC, 2007). A combination of dissolution and mixing combined with biological processes made the

ocean absorb about half of the CO_2 emitted since the beginning of the industrialisation (Sabine et al., 2004). Due to the reaction of CO_2 with water, the surface oceans' pH has already decreased by ~0.1 units and will continue to drop by additional 0.3 to 0.4 units until 2100 under a business-as-usual CO_2 emission scenario (IPCC IS92a; Meehl et al., 2007).

As photosynthetic CO_2 fixation is substrate limited under current atmospheric CO_2/O_2 ratios all photoautotrophic organisms evolved active carbon concentrating mechanisms (CCM), providing elevated [CO_2] at the site of carboxylation. In seawater, CO_2 concentration represents less than 1% of the inorganic carbon species and does indeed limit photosynthetic carbon fixation rates (Giordano et al., 2005). Elevated CO_2 concentrations are suggested to reduce the energetic costs for CCM (Fridlyand et al., 1996) and the regeneration of oxidised carbon acceptors and should thereby facilitate other energy consuming processes (Raven and Johnston, 1991; Riebesell, 2004). Indeed, rising CO_2 concentrations have been shown to enhance carbon fixation in several single species experiments (Riebesell, 2004; Hinga, 2002) and in natural plankton communities (Hein and Sand-Jensen, 1997; Riebesell et al., 2007).

Elevated nitrogen and carbon fixation rates mostly accompanied by enhanced cell division under projected future CO_2 conditions were measured in the filamentous oceanic cyanobacterium *Trichodesmium* (Barcelos e Ramos et al., 2007; Hutchins et al., 2007; Kranz et al., 2009; Levitan et al., 2007), as well as in the unicellular picocyanobacterium *Crocosphaera* (Fu et al., 2008). A combined positive effect of CO_2 enrichment and temperature on cell division rates was observed for the non-nitrogen fixing *Synechococcus*. But, under the same conditions *Prochlorococcus*, another picocyanobacterium, did not show this response (Fu et al., 2007).

Cyanobacteria can be found in a wide range of environments and are successful competitors even under conditions where inorganic carbon becomes ultimately limiting to primary production. This commonly occurs under high growth densities as prevailing in microbial mats and surface scums (Oliver and Ganf, 2000; Shapiro and Wright, 1990). Depending on the conditions in the natural habitats, CCM expression and activ-

ity can strongly differ between different species of cyanobacteria (Badger et al., 2005). While the expression of some components of the CCM appears to be constitutive, others were shown to be regulated in response to environmental factors (Beardall and Giordano, 2002; Shibata et al., 2001).

The stoichiometric composition of a mono-specific culture can show highly dynamic changes due to its ability to store nutrients in internal pools (Arrigo, 2005). A strong tendency to store nutrients is typical for cyanobacteria, especially if they are exposed to high fluctuations in nutrient supply (Allen, 1984). CO_2 related shifts in elemental ratios were also found in the studies on *Trichodesmium, Crocosphera* and on *Synechococcus* (Barcelos e Ramos et al., 2007; Hutchins et al., 2007; Fu et al., 2007; Fu et al., 2008). The biomass composition of cyanobacteria in these experiments as well as of seven microalgal species examined by Burkhardt et al. (1999) reacted differently to rising CO_2. Observed reactions in elemental ratios included increasing, decreasing and constant C/N and C/P ratios. These results imply that phytoplankton responses to future CO_2 concentrations will likely not follow a common pattern but may depend on the physiology of single species.

Exclusively in the Baltic Sea and in the Peel-Harvey estuary in Australia, the filamentous heterocystous cyanobacterium *N. spumigena* MERTENS, frequently forms extensive blooms that play a major role in the annual productivity of these regions (Sellner, 1997). Under calm conditions *Nodularia* accumulates at the surface forming big aggregates and even dense scums. Estimates of the annual nitrogen fixation of Baltic Sea cyanobacterial blooms are roughly equal to the total nitrogen input from river runoff and atmospheric deposition overall (Larsson et al., 2001; Schneider et al., 2003). Therefore *Nodularia* is of high biogeochemical importance for this region. In the Baltic Sea total inorganic carbon (DIC), salinity and alkalinity are lower compared to ocean values due to a strong riverine influence in this marginal sea. As a result of that and of a high biological productivity, the carbonate system shows a much stronger diurnal and seasonal variability than that of the open ocean, with strong temporal changes in pH, CO_2 and CO_3^{2-} concentrations (Thomas and Schneider, 1999).

In this study, aggregation is avoided as microclimate effects in the aggregates concerning the CO_2/O_2 conditions might cause significant alteration of CO_2 related physiological effects (Ploug, 2008). Carbon and nitrogen fixation of *Nodularia spumigena* were investigated under five different CO_2 concentrations. Simulated CO_2 conditions correspond to atmospheric concentrations between values in glacial periods and future values projected for 2100. Other environmental conditions were kept constant simulating a pre-bloom situation where single filaments are suspended within the surface layer.

We aim to determine whether the stimulating effects of elevated [CO_2] on carbon and nitrogen fixation found in *Trichodesmium* represent a general phenomenon among diazotrophic cyanobacteria and hence also apply to heterocystic *Nodularia*.

2 Material and methods

2.1 Setup

Semi continuous batch cultures of non-axenic *Nodularia spumigena* (IOW-2000/1) were grown at 16°C. This temperature was chosen because *Nodularia* blooms frequently develop in the southern Baltic Sea at this value (Kononen, 1992). The cultures were illuminated at an average intensity of 85 μmol photons $m^{-2} s^{-1}$ under a 14/10 h light/dark cycle. To ensure identical light conditions for all bottles, their positions were shifted daily. Aggregation of cell filaments and the development of microenvironments, in which growth conditions can deviate from those in the bulk medium, was avoided by keeping the cultures homogenously mixed at all times. This was achieved through a rotating device (Planktongravistat) that slowly rotated the incubation bottles orthogonally to their axis at a constant velocity of 1 rpm.

For acclimation of the cultures to the CO_2 treatments, pre-cultures were grown for 13 days (for replicates 1–9), respectively 18 days for the two high CO_2-concentration treatments (replicates 11–15) to reach similar cell densities at the start of the experiment.

Pre-culture incubation corresponded to about seven to eight cell generations. The experiment was started with an initial Chl-*a* concentration of 1 $\mu g\, l^{-1}$ in three replicate bottles per treatment. Start values of dissolved inorganic carbon (DIC), total alkalinity (TAlk) and cell counts were measured. All treatments were sampled after an incubation time of seven days, so that DIC consumption in the bottles was less than 3%.

2.2 Growth medium

An artificial seawater based medium (Kester et al., 1967) was prepared using modified YBC II nutrients (Chen et al., 1996) without inorganic nitrogen and with reduced phosphate (5.4 $\mu mol\, l^{-1}$). A salinity of 8 was chosen in correspondence with the origin of the culture (southern Gotland Sea) and because this is the salinity where intensive blooms of *Nodularia* are commonly observed (Kononen, 1992).

DIC concentration was adjusted to a value typical for the open Baltic Sea (1981 $\mu mol\, kg^{-1}$). After preparation, the medium was 0.2 μm sterile-filtered into one litre glass bottles. The media for the pre-culture and experiment were prepared from one batch.

2.2.1 CO_2 manipulation

Both TAlk and DIC were measured after filtration of the media. Based on these measurements, manipulation was carried out by adding HCl or NaOH to obtain an experimental pCO_2 range between ~150 and ~700 ppm and a corresponding pH range between 8.6 and 8.0 (on the free scale). The low end pCO_2 value of 150 ppm was chosen because at the time of *Nodularia* bloom development, i.e. early to mid summer, pCO_2 levels in the Gotland Sea are typically between 100 and 200 ppm (Thomas and Schneider, 1999). These comparatively low CO_2 levels result from intense biological activity earlier in the season combined with a low buffer capacity of the Baltic Sea brackish waters.

2.3 Measuring methods

2.3.1 Seawater carbonate system

DIC was measured after Stoll et al. (2001) in a QUAATRO analyzer (Bran and Lübbe GmbH, Norderstedt, Germany) equipped with a XY-2 sampling unit. The precision and accuracy of this method are ~2–3 μmol kg^{-1}. TAlk was determined in duplicate samples via potentiometric titration (Dickson, 1981) with 0.005 M HCl at 20°C in a Metrohm Tiamo automatic titration device (Metrohm GmbH & Co. KG, Filderstadt, Germany) with a precision of ~2 μmol kg^{-1}. The pH electrode of the alkalinity device was calibrated with pH buffers 4.0, 7.0 and 9.0 (Merck KgaA, Darmstadt, Germany). TAlk measurements were calibrated against Dickson seawater standard for CO_2 measurement (Marine Physical Laboratory, University of California, A. G. Dickson). pCO_2 and pH (on the free scale) were calculated from DIC and TAlk measurements with the program CO2SYS version 01.05 by E. Lewis and D. Wallace (for distinct treatment values see Table 1).

2.3.2 Chlorophyll-*a*

Chl-*a* concentrations were determined fluorometrically according to Welschmeyer (1994). Triplicates of 50 ml per bottle were filtered under a low vacuum of ~200 mbar on glass fibre filters (Whatman GF/F 25 mm Ø) and stored frozen at −18°C. Filters were homogenised in acetone and the extract measured in a Turner flourometer 10-AU (Turner BioSystems, CA, USA).

2.3.3 Particulate organic matter

Quantification of POC and PON was carried out via an elemental analyzer with a heat conductivity detector EuroVektor EA (EuroVektor S.p.A., Milan, Italy) according to Sharp (1974). 200 ml of sample were filtered at a pressure of ~200 mbar on a

combusted filter (Whatman GF/F 25 mm Ø) and subsequently stored at −18°C. Before measurement, filters were dried at 60°C for at least 5 h and packed into tin boats. Samples were calibrated against acetanilide C/N=10.36/71.09 kg/kg).

POP was determined following Hansen and Koroleff (in Grasshoff et al., 1983) adapted to the measurement of samples on glass fibre filters. 200 ml of sample were filtered at a pressure of ~200 mbar on combusted filters (Whatman GF/F 25 mm Ø) and subsequently stored at −18°C. Biomass was completely oxidised by heating the filters in 50 ml glass bottles with 35 ml of alkaline peroxodisulphate solution in a pressure cooker. Solutions were measured colorimetrically in a spectrophotometer U 2000 (Hitachi-Europe GmBH, Krefeld, Germany) with a precision of ~±0.2 μmol l^{-1}.

2.3.4 Nitrogen fixation

Nitrogen fixation was determined using the acetylene reduction assay with batch incubation technique according to Capone (1993) considering the Bunsen gas solubility coefficient determined by Breitbarth et al. (2004). Triplicate samples of 50 ml volume were transferred into gastight vials and purified acetylene was injected (~25% of the headspace volume). After four hours of incubation at a light intensity of 85 μmol photon m^{-2} s^{-1}, 300 μl of headspace were injected into a gas chromatograph (Shimadzu GC-14B; RT-Alumina? AL2_O3_Plot Column, Restek GmbH, Bad Homburg, Germany) with flame ionization detector. To convert acetylene reduction into nitrogen fixation, a conversion factor of three was used (Capone, 1993).

2.3.5 Cell counts

For determination of cell numbers, heterocyst frequency and cell dimensions, samples were filtered on white cellulose-acetate filters (25 mm Ø 1.2 μm pore size AE95 Schleicher and Schüll, Dassel, Germany) under low vacuum (200 mbar). Photographs were taken with an Observer A1 microscope and an AxioCam MRc (Carl Zeiss, Jena, Germany). Width and length of vegetative cells and heterocysts were measured using

the free computer program Image-J (Wayne Rasband, wayne@codon.nih.gov, NIH, Bethesda, MD, USA). For cell counts, duplicate samples of 30 ml were filtered and stored dry. 22–28 photos were taken systematically in a transect covering the diameter of the filter and additional 15 photos were taken randomly. The contrast of the photographs was altered by a macro (programmed by Manfried Ditsch) in Photo Shop software version CS3 (Adobe systems, San José, CA, USA) in order to achieve a (spreading) value based on a colour histogram that could then be correlated linearly with the surface covered by the cells. This spreading value was calibrated against exemplary cell counts that were estimated by using the computer program Image-J. Corrections were made considering the heterocyst frequency and dimensions (for cell count method see Czerny et al., 2009).

2.3.6 Statistics

Cell division rates (μ) were calculated according to:

$$\mu = \frac{\ln n_1 - \ln n_2}{\delta t} \quad (1)$$

(n_1=cell number at t_1, n_2=cell number at t_2, $\delta t = t_2 - t_1$)

Scatter plots were constructed using the program Statistica 6.0 (StatSoft Inc., Tulsa, USA). Each replicate bottle is represented by one data point. Regression lines represent a Pearson correlation with regression bands depicting the 95% confidence limits and determination coefficient r^2 for the fitted line. The p value is calculated from an f-test, testing the null hypothesis that the overall slope is zero and that there is no linear relationship between x and y. Normal distribution of data is assumed.

3 Results

Cell division rates differed significantly among treatments, reaching the maximum values of $\sim$0.52 d^{-1} at the lowest CO_2 level and the minimum values of $\sim$0.33 d^{-1} at

elevated CO_2 levels (Fig. 1a). This resulted in a total decrease in cell division rate of 36% over the experimental CO_2 range. A slight decrease in cellular nitrogen fixation in relation to rising pCO_2 was tested to be barely significant (p=0.014) (Fig. 1c). A significant increase in cellular carbon and phosphorus content with rising [CO_2] was detected (Fig. 2a, c). Taking the regression line as a mean, carbon and phosphorus cell quota increased from low to high CO_2 treatments by 32% and 30%, respectively, while cellular Chlorophyll-*a* did not change with CO_2 treatment (Fig. 1b). In contrast to carbon and phosphorus cell quota, the cellular nitrogen content did not show a clear trend with CO_2 (Fig. 2b). Rates of cellular carbon and phosphorus production (calculated from cell quota and cell division rate) did not show a significant trend over the experimental CO_2 range (Fig. 3a, c). A decreasing trend with CO_2 was obtained for nitrogen production derived from cell quota and division rate, comparable to and of similar statistical significance as measured nitrogen fixation rates (Fig. 1c), but with a steeper slope (Fig. 3B). Despite distinct differences in cell quota, no change in cell dimensions or heterocyst frequency could be detected in response to the CO_2 treatment. The mean length of vegetative cells was 3.8±0.46 μm (n=601), at a filament width of 10.7±0.91 μm (n=125). Heterocyst length was 8.8±1.2 μm (n=63). In all samples, one of 12 cells ±1 was a heterocyst.

Carbon to nitrogen ratios exhibited a highly significant (p<0.001) increase (26%) in response to elevated [CO_2] and lowered pH. At low [CO_2], C/N was about 5.5 and thus below the Redfield ratio while a maximum value (7.0) at high [CO_2] slightly exceeded the Redfield ratio (Fig. 4a). In contrast to this trend in C/N, the carbon to phosphorus ratio was not affected by changes in [CO_2], remaining constant at about 170, which is 58% above the Redfield value (Fig. 4b). Consistent with the different responses of nitrogen and phosphorus cell quotas, the N/P ratio showed a declining trend with increasing CO_2, complementary to C/N ratio (Fig. 4c).

4 Discussion

Various studies in recent years have demonstrated direct effects of rising CO_2 concentrations on cell division rates and/or carbon fixation in mono-specific cultures of eukaryotic (Burkhardt et al., 1999; Riebesell, 2004; Yang and Gao, 2003; Hinga, 2002) and prokaryotic (Barcelos e Ramos et al., 2007; Fu et al., 2007; 2008; Hutchins et al., 2007; Kranz et al., 2009; Levitan et al., 2007) marine phytoplankton. Results from lab-culture experiments are corroborated by studies on natural marine phytoplankton assemblages (Hein and Sand-Jensen., 1997; Riebesell et al., 2007), demonstrating that, in cases where a CO_2 effect has been detected, it resulted in the stimulation of cell division, carbon and nitrogen fixation. In contrast, in this study a mono-specific culture of *Nodularia spumigena* revealed a decrease in division rates in response to increasing pCO_2. Aside from the unexpected slope of this trend, a surprising observation also was that the inverse relationship of cell division rate and pCO_2 extended to a CO_2 partial pressure of 150 ppm, i.e. well below the pre-industrial level of 280 ppm. The fact that the trend did not level off towards the low CO_2 concentrations suggests that maximum cell division rate may occur at even higher pH and lower [CO_2] than tested here, values quite untypical for seawater.

The decrease in cell division rate with decreasing pH may be explained in the context of the natural growth conditions of *Nodularia* in seasonally or locally alkaline environments. These alkaline conditions are frequently caused when primary production results in a strong CO_2 drawdown in poorly buffered brackish water (Thomas and Schneider, 1999). Cyanobacteria in terrestrial habitats (i.e. lichens and microbial mats; Hallingbaeck, 1991) and in lakes are known to react in a similar way to acidification as *Nodularia* did in this experiment (Shapiro and Wright, 1990; Whitton and Potts, 2000). Acidification of different ranges (between pH 7.7 and 4.4) caused by anthropogenic atmospheric deposition of strong acids (H_2SO_4, HNO_3) substantially changed the phytoplankton communities of many lakes. Cyanobacteria were the only phytoplanktonic group that became nearly extinct in these acidified lakes (Findlay, 2003). Especially the

genus *Nostoc*, a close relative of *Nodularia,* is described by Mollenhauer et al. (1999) as an “endangered constituent of European inland aquatic biodiversity”. The explanation for the particular success of cyanobacteria under alkaline conditions is still unclear (Whitton and Potts, 2000).

With rising [CO_2], an increase of organic carbon and phosphorus within the cells could be detected while the accumulation of cellular nitrogen was less pronounced. Consequently a highly significant increase of the elemental C/N ratio was measured in the formed biomass. As cellular Chl-*a* content as well as production rates of carbon and phosphorus in organic matter did not seem to be negatively affected by the treatment, it is clear that cell division was neither limited by carbon fixation nor by energy supply. In fact, the accumulation of phosphorus and carbon in cellular reservoirs has to be seen as a result of the reduced division rate. The mechanism responsible for the observed pH/CO_2 sensitivity of cell division rate is still unknown.

Assuming that reduced division rates alone resulted in the storage of nutrients that would have otherwise been distributed among daughter cells, a proportional storage of carbon, phosphorus and nitrogen with decreasing cell division rate would have been expected. However, in this experiment, cellular carbon and phosphorus content showed a much stronger increase with rising experimental [CO_2] than cellular nitrogen content. As the observed decrease in cellular nitrogen fixation rates was not strong enough to account for the strong shifts in N/P ratios, impeded nitrogen transfer from heterocysts to vegetative cells seems to be the most reasonable explanation.

While in the non-heterocystic cyanobacterium *Trichodesmium* (Barcelos e Ramos et al., 2007; Hutchins et al., 2007; Kranz et al., 2009; Levitan et al., 2007) increased nitrogen fixation rates with rising CO_2 levels were interpreted to benefit of surplus energy from photosynthesis, in the present study nitrogen fixation in the heterocystous *Nodularia* was not enhanced by the treatment. In heterocystous cyanobacteria, nitrogen fixation is spatially separated from the bulk of photosynthetically derived energy. The energy (ATP) and the reductive power (NADPH, ferredoxin) for nitrogen fixation in heterocysts are partly derived from cyclic electron transfer in photosystem I inside the

heterocysts and partly from the pentose phosphate cycle that is supplied with carbohydrates from adjacent vegetative cells (Wolk et al., 1994). Heterocysts are probably not directly affected by [CO_2] as they do not fix CO_2 themselves because they lack photosystem II and RUBISCO (reviewed in Böhme, 1998). However, a pH effect on exchange processes between heterocysts and vegetative cells seems possible. A defective communication between heterocysts and vegetative cells under low pH could provide an explanation for the relatively weaker accumulation of particulate organic nitrogen compared to carbon and phosphorus. Nitrogen fixed by heterocysts is transferred to vegetative cells by high affinity (active) and low affinity (passive) transport of amino acids (Montesinos et al., 1995). Among others, the acidic amino acid glutamic acid and the basic amino acid arginine play major roles as vehicles of fixed nitrogen out of heterocysts and into the vegetative cells (Böhme, 1998). This exchange occurs mainly by diffusion between the end membranes of two adjacent cells, therefore amino acids have to pass the external media (Wolk et al., 1994). Due to the ion charge, weak acids can pass transporters only in the protonated form and weak bases can pass only in the deprotonated form. Thus, the transport of weak acids and bases shows a high sensitivity to pH differences across the cell membrane (Decoursey, 2003). Based on the findings in pH dependent cell division rate one may speculate that *Nodularia* is, also concerning its intracellular pH, adapted to the temporal occurrence of basic microenvironments. Assuming a rather constant internal pH of the cells, the uptake of basic substances like arginine, in more acidic environment, would be hindered in vegetative cells. Simultaneously, an accumulation of metabolites in heterocysts due to an impeded release of acidic compounds like glutamic acid because of an adverse proton gradient across the cell membrane is possible. This could cause an unbalance in the metabolism of heterocysts that could provide an explanation to the slight decrease in nitrogen fixation rates under low pH. The regulation of heterocyst differentiation by the availability of fixed nitrogen has been demonstrated in several studies (see references in Wolk et al., 1994). The constant heterocyst frequency observed in this study could be seen as an indication of no limitation by the supply of fixed nitrogen to vegetative

cells, but may also be the maximum frequency differentiated in N-free growth media.

When calm weather leads to an accumulation of *Nodularia* on the surface, high irradiance and associated high photosynthetic activities are likely to promote high nitrogen fixation rates (Paerl et al., 1985). A study of Surosz et al. (2006) showed that *Anabeana flos-aquae,* a relative of *Nodularia* with a similar autecology, reacts to nitrogen starvation with enhanced aggregation due to increased production of transparent exopolymer particles (TEP). In another study, *Anabaena flos-aquae* agglomerated in layers several centimetres thick was shown to exhibit higher nitrogenase activity than in dispersed filaments, despite high [O_2] caused by photosynthesis (Kangatharalingam et al., 1991). Hereby the ratio between nitrogen fixation and carbon fixation in filaments outside aggregation was lowest and increased towards the centre. Additionally, it seems reasonable that nitrogen storage is enhanced within surface scums also as the microenvironment is enriched with amino acids emitted from heterocysts of neighbouring filaments. In summary, there are many hints supporting the hypothesis that aggregation of heterocystic cyanobacteria is a strategy to improve nitrogen fixation and storage. In contrast, in this study accumulation of *C* and *P* in cellular reservoirs was found in the constantly dispersed *Nodularia* filaments. In nature phosphorus and carbon are in short supply within the surface scum, while short mixing events may provide the possibility for bacteria to store phosphorus and carbon. This ecological scenario could give a possible explanation for the observed pH preference and the strong accumulation of phosphorus and carbon relative to nitrogen in a homogeneous non-agglomerated culture.

Aggregation in clusters and microbial mats is a phenomenon observed for many planktonic, benthic and terrestrial cyanobacteria. In the Baltic Sea, *Nodularia* is infamous for forming dense toxic surface scums that cause considerable nuisance along the coastlines every summer. *Nodularia* often dominates the cyanobacterial community under relatively calm weather conditions, when aggregate formation is most prominent. When turbid conditions or storms interrupt calm weather, picocyanobacteria and other filamentous species that are usually more dispersed in the water column take

over dominance (Kononen, 1992; Sellner, 1997; Stal et al., 2003). Hence, it appears that *Nodularia* profits from the physical or chemical microenvironment prevailing in surface aggregations. Little is known about whether and how cyanobacteria benefit from this fact. There are only a few studies showing that aggregation can be a purposeful process in cyanobacteria (Ohmori et al., 1992; Koblížek et al., 2000).

In surface scums, especially in poorly buffered brackish waters like the Baltic Sea (Thomas and Schneider, 1999), pH can rise several units and DIC can be significantly lowered due to high photosynthetic demand for CO_2. Alternating conditions of pH 9 at daytime and pH 7 in darkness were measured inside *Nodularia* aggregations (Ploug, 2008). According to several authors (Oliver and Ganf, 2000; Shapiro and Wright, 1990), cyanobacteria outcompete eukaryotes under high pH and low CO_2 conditions of freshwater blooms. Observations that dispersed *Nodularia* filaments showed no CO_2 fertilising effect, as seen for *Trichodesmium*, could indicate that *Nodularia* possesses a similar ecological strategy as their freshwater relatives. A high affinity CCM apparatus that would allow *Nodularia* to outcompete other phytoplankton in a CO_2 limited microenvironment of a dense cyanobacterial bloom is possibly an ecological specialisation that can not be down regulated sufficiently to profit from [CO_2] as high as applied in this study.

After all, an explanation for the reduced division rates at pH-values commonly found in seawater can not be given. This emphasises that there is an urgent need to investigate pH dependent mechanisms that could be responsible for the observed effects. It can be speculated that the pH optimum found in *Nodularia* is an adaptation to the chemical microenvironment caused by photosynthesis in aggregations. But how and whether aggregated *Nodularia*, adapted to diurnal pH variations ranging from 7 to 9 (Ploug, 2008), react to comparably small changes caused by an atmospheric [CO_2] increase of some hundred ppm is questionable. Considering a pH of 9 inside the aggregations it is conceivable that a more acidic surrounding, as it is projected for the future, could cause a relief to problems in carbon acquisition. Aggregation is the crucial factor controlling the chemical environment of many important aquatic diazotrophs

and has to be included when assessing future oceanic carbon and nitrogen fluxes.

In this study it is clearly shown that under pre-bloom conditions, with single filaments dispersed in the upper water column, cell division is highly negatively influenced by acidification. Reduced division rates with rising atmospheric pCO_2 in the pre-bloom phase could lead to a progressive delay in the formation of the characteristic aggregates and thus of the initiation of *Nodularia* blooms. As the development of *Nodularia* is delayed, it could be outcompeted by other phytoplankton species that are either less or positively affected by rising [CO_2]. For *Anabaena*, a cyanobacterium often found together with *Nodularia*, there is indication that there will be a different reaction in response to rising [CO_2] (Franz et al., 2009). Since cyanobacterial blooms in the Baltic Sea are always composed of different species, it is probable that there will be a gradual change in species composition. Therefore, a CO_2 related decrease of the Baltic Sea nitrogen budget cannot be postulated. Carbon export is more likely to be enhanced if *N. spumigena* is replaced by other species since it is known that *N. spumigena*, due to the buoyancy of persisting gas vacuoles and living filaments, decomposes largely in the upper water column (Hoppe, 1981; Sellner, 1997).

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Table 1. Values of the carbonate system in the 14 experimental units. Alkalinity was measured in the end of the experiment while DIC was determined at the beginning and in the end. pH values (on the free scale) and CO_2 partial pressures are calculated for the point at which half of the DIC consumed during the experiment has been taken up.

Replicate	pCO_2 (ppm)	pH	Alkalinity (μmol kg^{-1})	DIC Start (μmol kg^{-1})	DIC End (μmol kg^{-1})
1	162	8.55	2188	1981	1946
2	153	8.57	2193	1981	1933
3	154	8.57	2191	1981	1933
4	297	8.31	2090	1981	1943
5	295	8.31	2091	1981	1945
6	313	8.29	2088	1981	1950
7	446	8.14	2051	1981	1959
8	435	8.15	2051	1981	1954
9	459	8.13	2047	1981	1957
11	508	8.08	2022	1981	1931
12	532	8.06	2019	1981	1934
13	723	7.98	1985	1981	1923
14	697	7.94	1987	1981	1920
15	731	7.93	1986	1981	1926

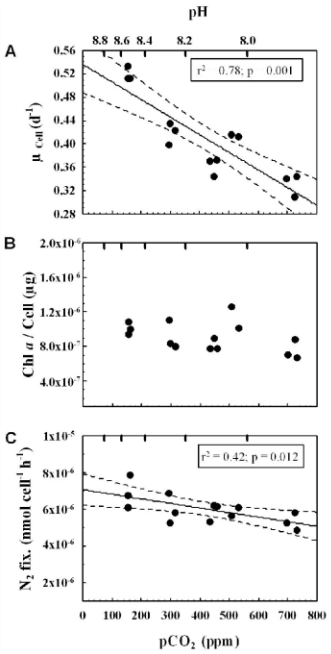

Fig. 1. Cellular division rate **(a)**, cellular chlorophyll-*a* content **(b)** and nitrogen fixation rate **(c)** as a function of CO_2 partial pressure and corresponding pH. Each data point represents one bottle. For the regression line (solid) regression coefficient r^2 and p value are given in the box. The dashed line represents a confident interval of 95%.

4301

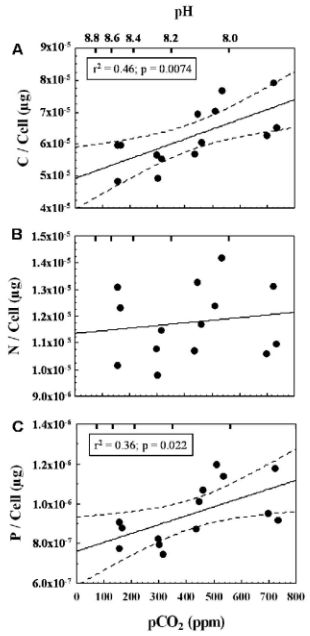

Fig. 2. Variations in cell quota of carbon **(a)**, nitrogen **(b)** and phosphorus **(c)** as a function of CO_2 partial pressure and corresponding pH. Each data point represents results from one bottle. For the regression line (solid) regression coefficient r^2 and *p* value are given in the box. The dashed line represents a confident interval of 95%.

4302

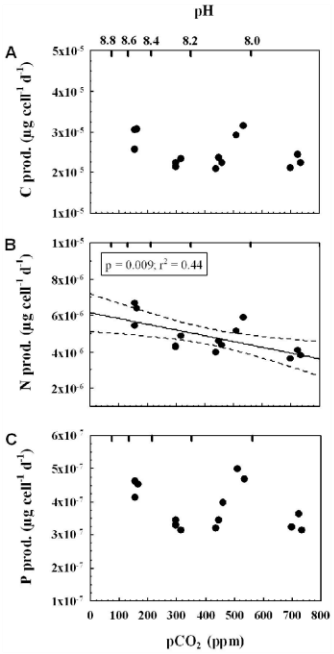

Fig. 3. Cellular production rates of POC **(a)**, PON **(b)** and POP **(c)** as a function of CO_2 partial pressure and corresponding pH. Each data point represents one bottle. For the regression line (solid) regression coefficient r^2 and p value are given in the box. The dashed line represents a confident interval of 95%.

4303

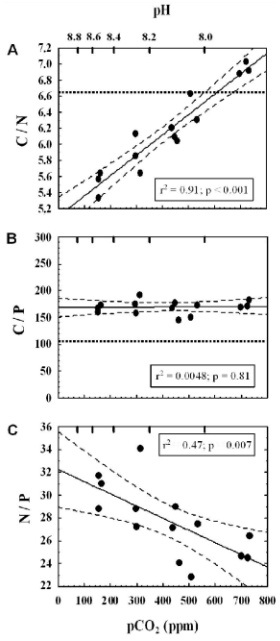

Fig. 4. Variation in C/N **(a)**, C/P **(b)** and N/P **(c)** molar ratios as a function of CO_2 partial pressure and corresponding pH. Each data point represents results from one bottle. The dotted lines mark the Redfield ratio. For the regression line (solid) the regression coefficient r^2 and p value are given in the box. The dashed line represents a confident interval of 95%.

4304

V Impact of CO_2 on the filamentous Baltic Sea cyanobacterium *Anabaena sp.*

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Manuscript in preparation

Impact of CO_2 on the filamentous Baltic Sea cyanobacterium *Anabaena spec.*

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Extensive summer blooms of filamentous cyanobacteria in the Baltic Sea are sustaining the biological production of this ecosystem. Potential changes in the extent of fixed nitrogen input affect microbiotic production, thus altering the biogeochemical fluxes within the Baltic Sea. In view of progressing ocean acidification, it is of great concern to investigate the effect of elevated CO_2 concentrations on marine diazotrophs.

Therefore, we studied the influence of the pCO_2 on *Anabaena spec*., one of the most dominant filamentous cyanobacteria in the Baltic Sea. *Anabaena* was confronted with pCO_2 levels ranging from glacial over present to predicted future atmospheric CO_2 concentrations. Growth rates of Anabaena stayed constant over the entire CO_2 range, whereas increases in the organic carbon-, nitrogen- and phosphorus-stoichiometry as well as in the total concentration of particulate organic carbon (POC) and nitrogen occurred with rising pCO_2. Further evidence for a CO_2 induced stimulation of the carbon fixation was the increasing DIC consumption per unit of POC in the course of elevated pCO_2. In contrast, nitrogen fixation rates per unit of biomass (POC, chlorophyll *a*) showed a decreasing trend towards a high CO_2 scenario, and thus, responded quite differently to CO_2 enrichment compared to most of the marine diazotrophs tested so far.

4. Synthesis

Mankind actions have been increasing atmospheric CO_2 concentrations since the industrial revolution and those are expected to almost triple from pre-industrial values by the year 2100, for a "business-as-usual" CO_2 emission scenario [*IS92a, IPCC*, 2007]. CO_2 enters the ocean by atmosphere - surface ocean gas exchange, decreasing carbonate ion (CO_3^{2-}) concentrations and pH in the surface ocean (ocean acidification). Additionally, the rise of CO_2 and other greenhouse gases is increasing global average temperatures in the atmosphere and consequently in the surface oceans. This establishes a thermal stratification, decreasing mixed layer depth and changing light supply. In this thesis emphasis was put on the potential impacts of global change on phytoplankton, both by varying CO_2 concentrations and light intensity. The first experiments were designed and performed when several scientific groups started wondering about the response of N_2-fixing cyanobacteria to increasing CO_2 concentrations. Results with the important nitrogen fixer *Trichodesmium sp.* presented here were published at about the same time as two other papers on the same subject (one group from Israel- *Levitan et al.*, 2007 and one from USA, *Hutchins et al*., 2007). All papers identified pronounced effects of rising CO_2 on this species. Because of that, work was developed with other filamentous cyanobacteria (*Nodularia spumigena* and *Anabaena sp.*) to assess whether the response is a general phenomenon in diazotrophs (co-authored manuscript IV and V).

The idea for the next study evolved from a growing discussion on the relevance of measurements done with acclimated cultures versus long-term incubations and whether starting samples from mesocosm studies reflect merely a stress effect after rapid changes in carbonate chemistry or a stable physiological response to a certain CO_2 concentration. Thus, manuscript II investigates how quickly *Emiliania huxleyi*, after being abruptly exposed to increasing CO_2 concentrations, shows inorganic and organic carbon fixation rates similar to those found in acclimated cultures.

Finally, manuscript III provides new information on how different species (*Emiliania huxleyi* and *Phaeodactylum tricornutum*) respond to abrupt changes in light conditions. This is of interest, since there might be stronger stratification in the future ocean and therefore changes in the light regime.

4.1. Responses of the non-heterocystous *Trichodesmium sp.* and the heterocystous *Nodularia spumigena* and *Anabaena sp.* to increasing CO_2 concentrations

Anthropogenic CO_2 has been taken up by the surface ocean, creating a decreasing vertical gradient with depth [*Sabine et al.*, 2004]. Thus, species occurring in the surface ocean like autotrophic nitrogen fixers (diazotrophs) could be the first organisms within the marine nitrogen cycle to be affected by increasing CO_2 concentrations. These key players in elemental cycling (as shown in section 2.3.2.) provide a nitrogen source to large areas of the ocean, fuelling primary production. Such considerations encouraged the evaluation of the sensitivity of the bloom forming diazotroph in the oligothrophic tropical and subtropical oceans [*Capone et al.*, 2005; *Mahaffey, Michaels & Capone*, 2005] *Trichodesmium* to expected rising CO_2 concentrations (publication I). From glacial CO_2 levels to those projected for the year 2100 there was a doubling of *Trichodesmium* cell division rate, decreasing cell dimensions with concomitant reduction of cellular carbon (C), nitrogen (N) and phosphorus (P) quotas. Moreover, nitrogen fixation rates per unit of phosphorus utilization more than doubled under enhanced CO_2 concentrations. Not only N:P increased but also C:P with no change in C:N. This indicates that carbon fixation was enhanced similarly to nitrogen fixation.

A similar response was found in more recent work with the unicellular diazotroph *Crocosphaera* [*Fu et al.*, 2007]. Under increasing CO_2 concentrations and replete nutrient conditions *Crocosphaera* also increased nitrogen and carbon fixation rates as well as N:P and C:P, with no trend in C:N. This species fixes nitrogen at night and carbon during the day, while in *Trichodesmium* both occur during the day but not at the same time. Although these species have distinct temporal occurrence of carbon and nitrogen fixation both processes occur in the same cell.

The explanation for such a strong response of these cyanobacteria may well be correlated to the down-regulation of the CO_2 concentrating mechanisms (CCMs) under enhanced CO_2 concentrations, either due to decreased diffusive loss of CO_2 from the cell and / or an increase in the proportion of diffusive CO_2 uptake into the cell. A down-regulation of the CCM activity might have increased the availability of energy and reductive power for other competing processes such as carbon and nitrogen fixation. In cyanobacteria the energetic gain at elevated CO_2 might be higher than in other

phytoplankton species due to the lower affinity to CO_2 in relation to O_2 of cyanobacterial Rubisco [*Tortell*, 2000].

Even though the non-heterocystous *Trichodesmium* and the unicellular *Crocosphaera* were affected in a similar way, the response of heterocystous species (heterocysts are morphologically differentiated cells) might be influenced by the separation of nitrogen and carbon fixation in space. Indeed, *Nodularia spumigena* and *Anabaena sp.* (co-authored manuscripts IV and V) do not respond to the increase in CO_2 concentrations like *Trichodesmium*. *N. spumigena* decreased cell division rate and *Anabaena sp.* was not affected under rising CO_2. Nitrogen fixation slightly decreased per phosphorus utilization in both heterocystous species. Moreover, strong changes in the cellular elemental composition occurred in *N. spumigena* [*Czerny, Barcelos e Ramos & Riebesell*, 2009], but uncertainties still exist regarding the response of *Anabaena sp.* [*Franz et al*., in preparation]. *N. spumigena* decreased N:P, maintained C:P and increased C:N indicating that carbon and nitrogen fixation are affected differently by the increasing CO_2 concentrations when compared to *Trichodesmium*. Cellular carbon, nitrogen and phosphorus increased due to the decreasing cell division rate with rising CO_2 concentrations. However, cellular nitrogen quota didn't increase as much as carbon and phosphorus, becoming evident from the C:N and N:P. This cannot be fully explained by the slight decrease in nitrogen fixation rate. *Czerny Barcelos e Ramos & Riebesell*, [2009] hypothesized as an explanation a reduced transport of nitrogen between heterocysts and vegetative cells. The mechanisms behind nitrogen transfer are still unclear but, as we shall see, a closer look on the characteristics of heterocystous species might help understanding differences observed.

Heterocystous cyanobacteria such as *Nodularia spumigena* and *Anabaena sp.* have specialized cells (heterocysts) with an additional glycolipid and a polysaccharide layer surrounding the outer membrane, forming a thick wall with low permeability to gases [*Flores & Herrero*, 2005]. The oxygen-sensitive nitrogen fixation occurs in these cells in almost anaerobic conditions since these cells do not have active PSII which would produce oxygen [*Wolk, Ernst & Elhai*, 1994] and the existing O_2 is consumed mostly in respiration. In *Anabaena* species, heterocysts are 7 % of the cells [*Böhme*, 1998], meaning that the fixed nitrogen has to pass several adjacent cells (about 7 cells) or the compound is released and then assimilated by vegetative cells. The fixed nitrogen is transported from the heterocysts to the adjacent cells [*Montesinos, Herrero & Flores*, 1995] in the form of amino acids, mostly glutamine [*Wolk, Ernst & Elhai*, 1994].

Moreover, heterocysts receive carbohydrates as sucrose from the vegetative cells [*Curatti, Flores & Salerno*, 2002; *Wolk, Ernst & Elhai*, 1994]. Although part of the fixed nitrogen might be exuded as reported for *Trichodesmium* [*Mulholland, Bronk & Capone*, 2004; *Capone, Ferrier & Carpenter*, 1994; *Glibert & Bronk*, 1994] probably the majority is exchanged to the vegetative cells without direct contact with the exterior. Once it is taken up by a vegetative cell it can easily be assimilated by other adjacent cells due to the presence of a shared space continuum between the outer and inner membranes, the periplasmic space, as shown in *Anabaena sp*. [*Flores et al.*, 2006]. The assimilation of amino acids that were exuded has been hypothesized to be affected by decreasing pH [*Czerny, Barcelos e Ramos & Riebesell*, 2009]. Whether the pH of the periplasmic space is influenced by extracellular pH is difficult to predict at this point. In any case, the potential pH sensitivity of amino acids transport [*Decoursey*, 2003] could explain the lower cellular nitrogen contents. Moreover, if transporters performing assimilation of amino acids in vegetative cells are affected by a pH decrease, then transporters responsible for exudation of these compounds in heterocysts might also be influenced. This could then cause the slow down of nitrogen fixation rates in *N. spumigena* and *Anabaena sp*. in contrast to what was seen in the non-heterocystous species. The reason for the lack of carbon production stimulation remains unclear. However, it might come as a consequence of nitrogen limitation in the vegetative cells due to the hindered transport of amino acids between those cells and heterocysts.

The reason for the differences found within the heterocytous species *N. spumigena* and *Anabaena sp.* are still unknown. These species have very different cell sizes, with *Nodularia spumigena* being much bigger. This changes their area to volume ratio influencing the transfer efficiency of for instance CO_2 and amino acids. Since cyanobacteria have the ability to store nutrients internally, especially when subjected to varying nutrient supplies [*Allen*, 1984], the cell size might influence cellular quotas.

The increase of nitrogen fixation rates per phosphorus utilization as seen in *Trichodesmium* and *Crocosphaera* could favour productivity of N-limited oligotrophic oceans for a certain period of time, potentially quickly driving some of these areas into phosphorus limitation, and increase biological carbon sequestration in the ocean. Thus, the observed CO_2 sensitivity of *Trichodesmium* could act as a negative feedback to the expected CO_2 increase. Opposing to that, if the slight decrease in nitrogen fixation of the heterocystous species studied to date could be generalized to other heterocystous it could

provide a positive feedback to atmospheric CO_2. Nevertheless, the distribution and overall abundance of heterocystous species is less significant than non-heterocystous ones.

However, the predicted rise in CO_2 concentration could also change the phytoplankton community structure in accordance with the competitive fitness of other co-occurring species (including diazotrophs). Preliminary results from experiments conducted with a Baltic Sea community during a diazotrophic bloom exposed to changing CO_2 at varying and constant pH (data not shown), did not show a marked response of nitrogen fixation rates. It is still unclear whether this was the result of an undetectable response of the dominant species or a sum of different responses from various diazotrophs.

4.2. Response of the coccolithophore *Emiliania huxleyi* to abrupt CO_2 changes

Changes in the ocean's CO_2 levels are predicted to occur at an unprecedented rate. Most studies done to date to test the effects of increasing CO_2 concentrations on coccolithophores used cultures pre-exposed to the experimental conditions, often considering an acclimation period of about 9 to 12 generations [*Feng et al.*, 2008; *Riebesell et al.*, 2000; *Zondervan, Rost & Riebesell*, 2002]. However, how long phytoplankton cells actually take to acclimate was virtually unknown. In manuscript II it is shown that the short-term response (hours to one day) of *Emiliania huxleyi* to CO_2 concentrations increasing from glacial (190 µatm) to projected year 2100 (750 µatm) levels is similar to that obtained with acclimated cultures under comparable conditions in earlier studies [*Riebesell et al.*, 2000]. Organic carbon fixation increased and calcification decreased 8 h after exposing the cultures to the changed conditions, with a concomitant decrease in the ratio of $CaCO_3$ to organic carbon production. This fast change in the rates of various essential processes and the establishment of a new physiological "state" which overall resembles acclimated cultures (cells exposed to the conditions for about 12 generations, about 10 days) showed that studies using acclimated cultures measured phenotypic plasticity of the individuals. However, in some species phenotypic plasticity of the individuals might not provide competitive fitness and the populations will have to adapt or the species might become extinct [*Bell & Collins*, 2008].

Species might vary in their capacity to acclimate to new conditions, depending on their past and present habitats. *Emiliania huxleyi* appeared in the geological record 270 000 years ago [*Thierstein*, 1977]. Because it is such a recent species it evolved under low CO_2 variations on geological time scales (glacial / inter-glacial) in comparison to the projected changes and to other phytoplankton like cyanobacteria. More importantly, atmospheric CO_2 concentration is expected to more than double in the next 100 years at an unprecedented rate [*IPCC*, 2007], reaching values higher than those of the last 2 million years [*Hönisch et al.*, 2009]. In this context, two main questions challenge researchers working with the effects of climate change on phytoplankton: 1) Will species in their natural environment respond similarly to acclimated cultures when exposed to the same changes in the carbonate chemistry? 2) Can phytoplankton species, and for that matter *E. huxleyi*, adapt to the expected changes in the future ocean? Natural populations of *E. huxleyi* have considerable genetic variation [*Medlin et al.*, 1996]. However, it is unsure whether this variability and species-specific phenotypic plasticity will be enough to cope with the changes, speed up or delay evolution. Additionally, co-evolution of predators, pathogens and hosts, and species competition within a certain niche might play an important role. The major drivers of evolution are mutations, which might occur for instance during an illegitimate recombination [*Cavalier-Smith*, 2002]. Sex is responsible for the introduction of smaller variations during homologous recombination than mutations. Even with such a high cell division rate like phytoplankton, it is hard to introduce evolution potential because time is directly related to the number of homologous recombinations. The phenotypic plasticity of the individuals, measured in acclimated cultures, will influence selective pressure, delaying or speeding up evolution, and might be crucial in shifting community composition in the environment before single species are able to adapt. Therefore, our attempt to understand how organisms, namely phytoplankton, could respond to the expected changes in the ocean should consider both phenotypic plasticity and adaptation potential.

4.3. Responses of *Emiliania huxleyi* and *Phaeodactylum tricornutum* to abrupt light intensity increases

In the marine environment light is constantly changing. Either by shifting cloud cover or radiation focusing and defocusing by surface waves [*Dera & Stramski*, 1986]. Moreover, phytoplankton cells experience light variation while transported through the water column. Species react differently to light variations [*Falkowski*, 1980], with potential consequences to their distribution. Indeed, phytoplankton groups' distribution can be generally described according to light, nutrient and turbulence. Nitrogen fixing cyanobacteria occur in higher abundances in areas with low availability of nitrate, coccolithophores dominate the calm, nutrient poor, oceanic environment and dinoflagellates and diatoms generally dominate coastal areas where light variation is often sudden and, nutrient concentrations and turbulence generally high. Explanations for these patterns and for differences between species from the same group are partly-related to photoprotective capacity, its regulation and photosynthetic architecture [*Lavaud, Strzepek & Kroth*, 2007; *Wagner, Jakob & Wilhelm*, 2006]. For instance, when comparing coastal and oceanic diatoms it was observed that oceanic diatoms inhabit iron-poor regions because they have lower iron requirements [*Sunda, Swift & Huntsman*, 1991; *Sunda & Huntsman*, 1995] due to lower concentrations of photosystem I and cytochrome b_6f complex [*Strzepek & Harrison*, 2004], therefore having less efficient short-term energy dissipation [*Munekage et al.*, 2001].

Even though it is possible to define mechanisms responsible for the observed horizontal distribution of species, like presented above for diatoms, the reasons behind functional groups distribution such as coccolithophores and diatoms are not fully understood. In fact, the time coccolithophores and diatoms take to activate their energy dissipation mechanisms and how much of the extra energy can be utilized in photochemistry hasn't been investigated so far. In manuscript III the effect of an abrupt light increase on energy dissipating mechanisms, including photochemistry, in the diatom *Phaeodactylum tricornutum*, and the coccolithophore *Emiliania huxleyi*, were analysed. The photoprotective response could be detected through changes in the xanthophyll cycle, with the increase of both diadinoxanthin and diatoxanthin. The diatom changed its pigment contents faster than the coccolithophore. This quick formation of diatoxanthin in the diatom translates to a decreased photoinhibitory damage of PSII [*Ting & Owens*,

1994] due to the onset of non-photochemical quenching [*Kashino et al.*, 2002; *Lavaud et al.*, 2002; *Olaizola et al.*, 1994].

The abrupt increase in light intensity also rapidly increased organic carbon fixation in both *Phaeodactylum tricornutum* and *Emiliania huxleyi,* but faster in the diatom. That could be explained, at least in part, by the presence of a C_4 - like mechanism in the diatom which could fix CO_2 into an intermediate compound [*Reinfelder, Milligan & Morel,* 2004]. When light intensity increases this intermediate could rapidly be available for carbon fixation by Rubisco. *E. huxleyi,* initially fixed less organic carbon, but still dissipated considerable amounts of excess energy by calcification.

The observed results highlight the importance of response time. *P. tricornutum* showed the capacity to react at shorter time scales than *E. huxleyi* both in energy dissipation as heat and organic carbon fixation. Unexpectedly, *E. huxleyi* increased inorganic carbon fixation in the first minutes. Although the function of calcification is still unknown, this could hint at an important additional role of coccolith formation. Finally, CCMs might play an important role in compensating for variations in CO_2 demand after the light intensity increases, but its potential consequences for inorganic carbon fixation have been neglected. Moreover, if a C_4-like mechanism can be generalized to all diatoms, it might be an additional key process in a constantly changing environment, such as coastal areas.

Moreover, the build up of CO_2 in the atmosphere raises global average temperature [*IPCC,* 2007] together with surface ocean temperatures. This might strengthen water column stratification and decrease mixed layer depth, potentially creating a barrier to vertical mixing of nutrient-rich, deeper water [*Sarmiento et al.*, 2004]. Phytoplankton cells could experience under these conditions an increase of the average light intensity and more frequent high light exposure both in high and low latitudes. There are species - specific or possibly even functional group - specific responses to light intensity variation. Hence, a future ocean with reduced mixed layer depth could favor organisms able to respond faster to changes in light intensity and / or nutrient supply. This could influence communities composition, overall primary production, export of organic matter and ultimately carbon sequestration capacity. How much of the CO_2 taken up will be drawn down from the surface ocean through the carbon pump will depend on the actual export which at this point is hard to predict.

In low latitudes such as in the tropics the increase in light availability and decrease in nutrient availability have been speculated to have no major consequences for primary production. However, these regions are inhabited by nitrogen fixers who might provide

bio-available nitrogen to other phytoplankton species. Depending on the phosphorus available this could promote primary production in these regions until phosphorus limitation. A community dominated by diazotrophs could potentially affect the N:P of organic matter. Changes in the composition of organic matter might influence its decomposition and therefore drawdown of CO_2.

At high latitudes, especially in HNLC (high nutrients low chlorophyll) areas there could be an increase of primary production as long as micronutrients are available, due to an increase of light availability throughout the year. However, both temperature [*Daufresne et al.*, 2009] and CO_2 [*Tortell et al.*, 2002] changes could cause a shift in community composition towards smaller species; still work has to be done to confirm this.

4.4. Perspectives for future work

As it is often the case, from the answers found in this thesis several new questions emerged. In publication I the increase in CO_2 stimulated a strong response of cell division rate and both nitrogen and carbon fixation rates in *Trichodesmium*. It is hypothesized in the publication that the explanation for such a strong response might be the down-regulation of the CCM under high CO_2 concentrations, providing additional energy for other energy-demanding processes. It would be interesting to repeat the experiment but broaden the CO_2 range, since at high CO_2 concentrations the CO_2 surplus might be counterbalanced by the effect of correspondent low pH. The following step would be to investigate how this curve would shift under different temperatures. That is especially important because with the increase of sea surface temperature, concomitant stratification and decrease on nutrient availability, there might be an increase of nitrogen fixation [*Boyd & Doney*, 2002]. However, there could also be a decrease of the area where optimum nitrogen fixation and growth of *Trichodesmium* occur [*Breitbarth et al*., 2007].

Co-authored manuscripts IV and V revealed that heterocystous cyanobateria were affected differently by the rise in CO_2. *Czerny, Barcelos e Ramos & Riebesell* [2009] hypothesized that the reason for the drastic changes on the ratios could result from decreasing efficiency in the transport of amino acids between heterocysts and adjacent cells caused by a decreasing pH. Therefore, it would be most interesting to repeat the experiment with an additional set of cultures being exposed to rising CO_2 concentrations,

but at constant pH. Additional measurements of various amino acid concentrations could be used to test the hypothesis. Moreover, following the discussion about the role of aggregations on the response of filamentous cyanobacteria to increasing CO_2 concentrations, it is urgent to perform experiments with and without aggregate formation.

In manuscript II the response of *Emiliania huxleyi* to abrupt CO_2 variations revealed that non-acclimated cultures responded very similarly to acclimated cultures in a matter of hours. Hence, the time necessary for individual cells to establish a "new physiological state" in response to a change in environmental conditions (acclimation) was relatively short. This leads us to conclude that most of the experiments done to date testing ocean acidification are looking at individual's phenotypic plasticity. However, some species may depend on their ability to adapt to survive if the phenotypic plasticity of their individuals does not confer enough competitive fitness [*Bell & Collins*, 2008]. Thus, it is of major importance to also address the potential for adaptation of a certain species. Evolutionary studies done with the plant *Arabidopsis thaliana* [*Lau et al.*, 2007] and the alga *Chlamydomonas* [*Collins & Bell*, 2004] have shown that rising CO_2 induces phenotypic changes, but without signs of adaptation. Nevertheless, that does not mean that there is no potential for adaptation, perhaps rather that the experimental designs and species considered were not the most sensitive. It would be valuable to perform long-term experiments either with several clones, preferentially freshly isolated from the same location, and / or inducing sexual reproduction. Moreover, it would be important to have changing CO_2 concentrations rather than constant conditions, since the latter might force responses that in the natural variable environment wouldn't occur.

Considering the strong response of phytoplankton (shifting the peak of the bloom) and bacteria to changes in temperature [*Wohlers et al.*, 2009] it seems natural that further studies with both acclimated cells and long-term experiments combine CO_2 with other parameters such as temperature, light and nutrients.

In manuscript III the physiological effect of abrupt changes in light intensity on *Emiliania huxleyi* and *Phaeodactylum tricornutum* show that while the diatom can strongly increase organic carbon fixation in the coccolithophore it is inorganic carbon fixation that responds faster. This fast response is important in an environment that constantly changes as in coastal areas and perhaps, at least partly, explains the distribution of phytoplankton functional groups. However, there are also arguments for the importance of micronutrients availability such as iron in phytoplankton distribution [*Falkowski et al.*, 2004]. Hence, it would make sense to combine various light intensities

and iron concentrations in order to better understand the relative importance of these two variables in a group of species. Considering that calcification might function as an energy dissipation valve under abrupt increases of light intensity, it would be interesting to compare the responses of a non-calcifying and a calcifying *E. huxleyi* to those conditions.

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6. Snapshots of the PhD

Eidesstattliche Erklärung

Hiermit bestätige ich, dass die vorliegende Arbeit mit dem Titel:

Responses of selected species of marine phytoplankton to increasing carbon dioxide and light

von mir selbständig verfasst worden ist und keine weiteren Quellen und Hilfsmittel als die angegebenen verwendet wurden. Die vorliegende Arbeit ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft enstanden und wurde weder im Rahmen eines Prüfungsverfahrens an anderer Stelle vorgelegt noch veröffentlicht.
Ich erkläre mich einverstanden, dass diese Arbeit an die Bibliothek des IFM-GEOMAR und die Universitätsbibliothek der CAU weitergeleitet wird.

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Curriculum Vitae

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4. Publications

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