Biogeosciences, 6, 1865–1875, 2009
www.biogeosciences.net/6/1865/2009/

Biogeosciences

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

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Received: 1 April 2009 – Published in Biogeosciences Discuss.: 17 April 2009
Revised: 14 August 2009 – Accepted: 17 August 2009 – Published: 4 September 2009

Abstract. The surface ocean absorbs large quantities of the CO_2 emitted to the atmosphere from human activities. As this CO_2 dissolves in seawater, it reacts to form carbonic acid. While this phenomenon, called ocean acidification, 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.

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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 385 ppm (Keeling et al., 2008). 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 makes the ocean absorb about one third of the anthropogenic CO_2 emitted to the atmosphere (Sabine et al., 2004). Due to the reaction of CO_2 with water, the surface oceans' pH has already decreased by $\sim$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, see 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 (Hinga, 2002; Riebesell, 2004; Rost et al., 2008) and in natural plankton communities (Hein and Sand-Jensen, 1997; Tortell et al., 2002b; Riebesell et al., 2007).

Published by Copernicus Publications on behalf of the European Geosciences Union.

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; Levitan et al., 2007; Kranz et al., 2009), 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 (Shapiro and Wright, 1990; Oliver and Ganf, 2000). Depending on the conditions in the natural habitats, CCM expression and activity 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 (Shibata et al., 2001; Beardall and Giordano, 2002).

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; Fu et al., 2007, 2008; Hutchins et al., 2007). 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 *Nodularia spumigena*, frequently forms extensive blooms that play a major role in the annual productivity of these regions (Sellner, 1997). Aside from *Nodularia*, heterocystic filamentous *Anabaena* and *Aphanizomenon* often contribute considerably to the biomass of cyanobacterial blooms in the Baltic Sea. Under calm conditions *Nodularia* accumulates at the surface forming big aggregates and even dense scums. Estimates of annual nitrogen fixation in the Baltic Sea due to cyanobacterial blooms are roughly equal to the total nitrogen input from river runoff and atmospheric deposition (Larsson et al., 2001; Schneider et al., 2003). Therefore, *Nodularia* is of high biogeochemical importance for this region. In the Baltic Sea dissolved inorganic carbon (DIC), salinity and total 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 microclimatic effects on CO_2 and O_2 levels inside and outside the aggregations might induce a different physiological response to elevated pCO_2 than in dispersed filaments (Ploug, 2008). The experimental setup simulates a pre-bloom situation were single filaments are dispersed within the upper water column and aggregations have not yet formed. Carbon and nitrogen fixation of *Nodularia spumigena* were investigated under five different CO_2 concentrations. Simulated CO_2 conditions correspond to 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 irradiance of 85 μmol photons m^{-2} s^{-1} under a 14/10 h light/dark cycle, simulating the depth integrated irradiance of the mixed layer. 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, precultures were grown for 13 days (replicates 1–9), and 20 days for the two high CO_2-concentration treatments (replicates 11–15). Pre-cultures were grown in the same manner as the cultures in the experimental units. Different incubation periods were used in order to reach similar cell densities at the start of the experiment, despite the different division

rates. Pre-culture incubation corresponded to 7 to 8 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, during which CO_2 was drawn down by photosynthesis, as occures during the development of a bloom. DIC consumption in all bottles was less than 3%. The denoted [CO_2] and pH values were calculated for the point where half of the consumed DIC was taken up by the bacteria. TAlk was measured in the end of the experiment.

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 directly into the one litre glass culture bottles. To minimise gas exchange with air, filtration was performed by means of a peristaltic pump. The media for the pre-culture and experiment were prepared from one batch.

CO_2 manipulation

Both TAlk and DIC samples were taken from several culture bottles 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 7.9 (on the free scale). The acid/base manipulation technique was chosen here because it is the most simple and precise way for CO_2 manipulation in closed bottles without headspace. As the pCO_2 range applied in this experiment is relatively small, the carbon ion speciation compared to a method where DIC is changed while TAlk is kept constant, shows equal trends of similar magnitude (Schulz et al., 2009). Therefore, we do not expect significant differences in the measured results that are due to the manipulation approach. 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 & Lübbe GmbH, Norderstedt, Germany) equipped with a XY-2 sampling unit. The precision and accuracy of this method are ~2–3 $\mu mol\,kg^{-1}$. 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 $\mu mol\,kg^{-1}$. TAlk measurements were calibrated against Dickson seawater standard for CO_2 measurement (Marine Physical Laboratory, University of California, Prof. 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 ∅) and stored frozen at −18°C. Filters were homogenised in acetone and the extract measured in a Turner fluorometer 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 ∅) 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 ∅) and subsequently stored at −18°C. The 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 about ±0.2 $\mu mol\,l^{-1}$.

2.3.4 Nitrogen fixation

Nitrogen fixation was determined using the acetylene reduction assay with batch incubation technique according to

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

Capone (1993) and the Bunsen gas solubility coefficient determined by Breitbarth et al. (2004). Triplicate samples of 50 ml volume were transferred into gas-tight vials and purified acetylene was injected (∼25% of the headspace volume). After four hours of incubation at an irradiance 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 3 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 ⌀ 1.2 μm pore size AE95 Schleicher & 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. Twenty two to 28 photographs 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 in Photoshop software version CS3 (Adobe systems, San José, CA, USA) in order to achieve the 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.

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, δt=$t_2 - t_1$).

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.

3 Results

Cell division rates differed significantly among treatments, reaching the maximum values of ∼0.52 d^{-1} at the lowest CO_2 level and the minimum values of ∼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, while cellular Chlorophyll *a* did not change with CO_2 treatment (Fig. 1b). A slight decrease in cellular nitrogen fixation in relation to rising pCO_2 was tested to be barely significant (p=0.012) (Fig. 1c). A significant increase in cellular carbon and phosphorus content with rising [CO_2] was detected (Fig. 2a and c). Taking the regression line as a mean, carbon and phosphorus cell quota increased from low to high

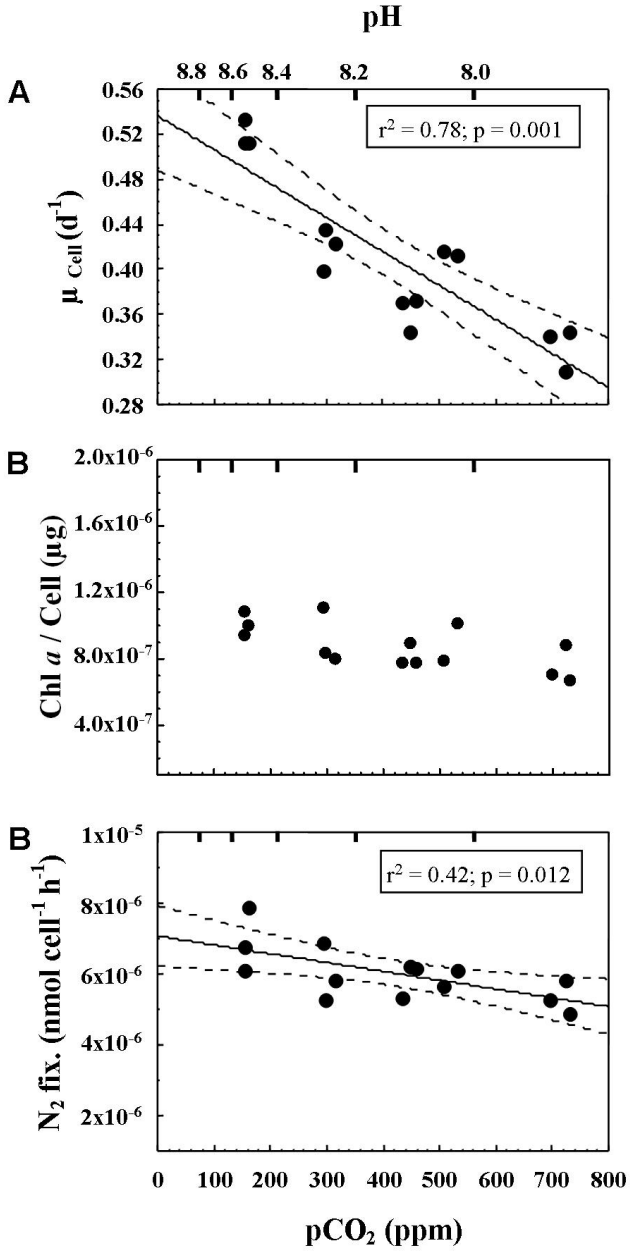

Fig. 1. Cellular division rate **(A)**, cellular chlorophyll *a* content **(B)** and nitrogen fixation rate **(C)** as a function of CO_2 partial pressure and corresponding pH (on the free scale). Each data point represents one bottle. The correlation coefficient r^2 and p value of the regression (solid line) are given. The dashed line represents the 95% confident intervals.

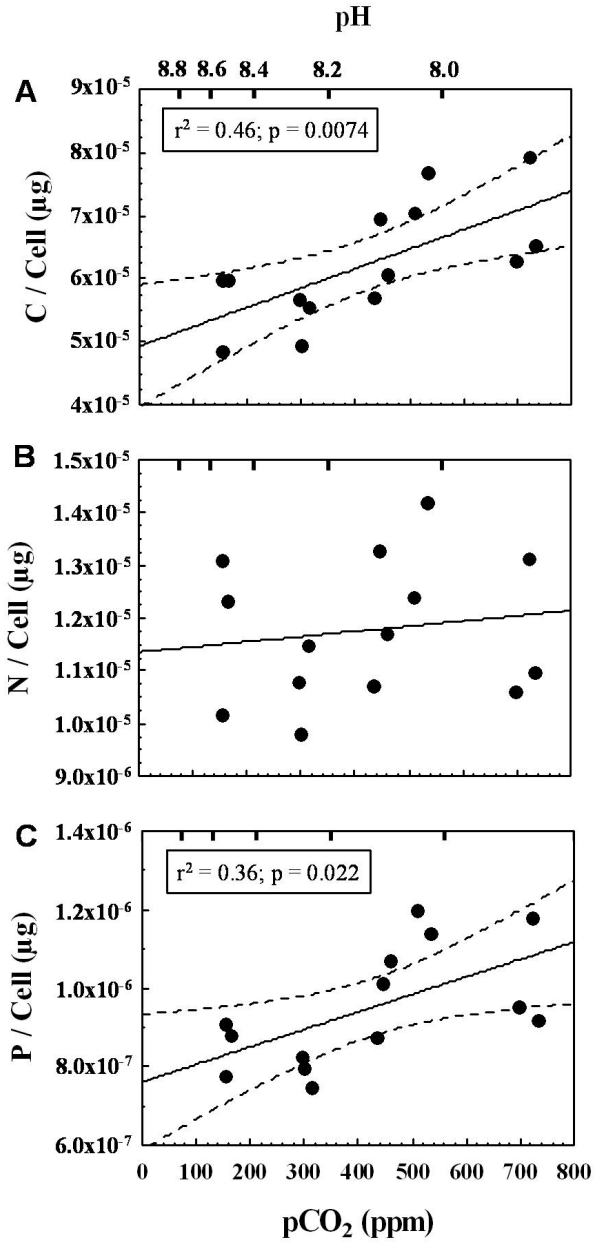

Fig. 2. Variations in cell quota of carbon **(A)**, nitrogen **(B)** and phosphorus **(C)** as a function of CO_2 partial pressure and corresponding pH (on the free scale). Each data point represents one bottle. The correlation coefficient r^2 and p value of the regression (solid line) are given. The dashed line represents the 95% confident intervals.

CO_2 treatments by 32% and 30%, respectively. 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 and 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.

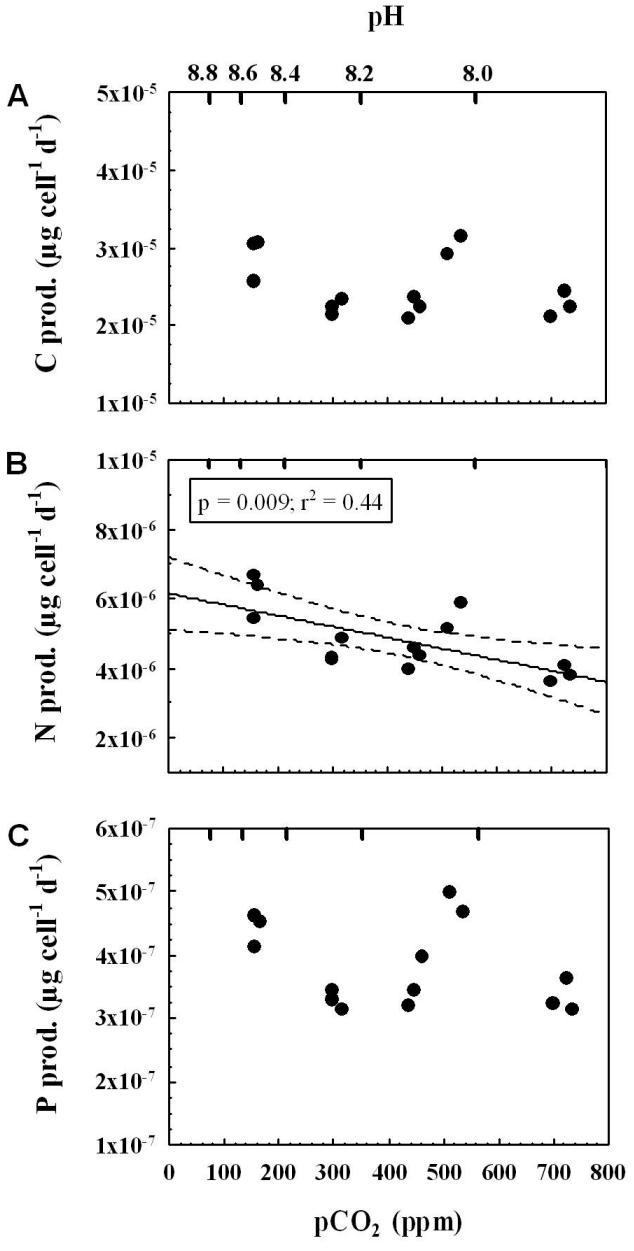

Fig. 3. Cellular net production rates of POC **(A)**, PON **(B)** and POP **(C)** as a function of CO_2 partial pressure and corresponding pH (on the free scale). Each data point represents one bottle. The correlation coefficient r^2 and p value of the regression (solid line) are given. The dashed line represents the 95% confident intervals.

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; Hinga, 2002; Yang and Gao, 2003; Riebesell, 2004) and prokaryotic (Barcelos e Ramos et al., 2007; Fu et al., 2007, 2008; Hutchins et al., 2007; Levitan et al., 2007; Kranz et al., 2009) marine phytoplankton. Results from 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 the present 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 cell size remained constant. Therefore, a substantial increase in cell density at elevated [CO_2] can be assumed (Allan, 1984;

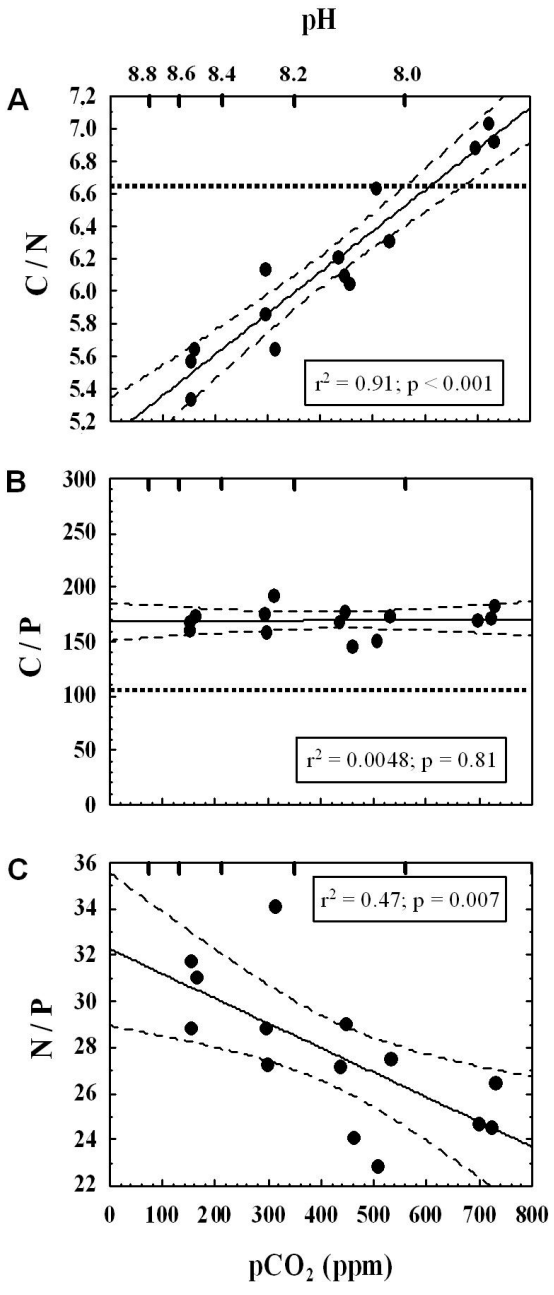

Fig. 4. Variation in C/N **(A)**, C/P **(B)** and N/P **(C)** molar ratios as a function of CO_2 partial pressure and corresponding pH (on the free scale). Each data point represents results from one bottle. The dotted line indicates the Redfield ratio. The correlation coefficient r^2 and p value of the regression (solid line) are given. The dashed line represents the 95% confident intervals.

Sellner, 1997). As cellular nitrogen content was not influenced by the treatment a highly significant increase of the elemental C/N ratio was measured in the formed biomass. As cellular Chl*a* content as well as the 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 is the result of the reduced division rate. The mechanism responsible for the observed pH/CO_2 sensitivity of cell division rate is still unknown.

If the reduced division rates alone resulted in the storage of nutrients that would have otherwise been distributed among daughter cells, storage of carbon, phosphorus and nitrogen proportional to the cell division rate would be 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 decrease in cellular nitrogen fixation rates was not large 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* 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 (Barcelos e Ramos et al., 2007; Hutchins et al., 2007; Levitan et al., 2007; Kranz et al., 2009). 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 transport 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 mainly occurs by diffusion between the end membranes of two adjacent cells (Wolk et al., 1994), therefore amino acids have to pass through the external media. 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 exhibits a high sensitivity to pH differences across the cell membrane (Decoursey, 2003). Based on the finding that cell division rate is pH dependent, 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, resulting in an increased loss rate of fixed nitrogen. 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 the present study could indicate that there was no limitation by the supply of fixed nitrogen to vegetative cells in all treatments. As the cultures were grown in N-free growth media, the observed frequency may also be the maximum number of heterocysts per vegetative cells that can be differentiated.

When calm weather leads to an accumulation of *Nodularia* at 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 at the outer areas of the 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 excreted by the 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 the present study C and P accumulated 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. A cross check of this study using a setup were filaments are allowed to aggregate would be the logical follow-up. Unfortunately, aggregates formed by cultured *Nodularia* bear little similarity to natural aggregations and can not be sampled adequately using quantitative methods as applied in this study. Therefore, an experiment of this kind is likely to deliver ambiguous results that may prove difficult to extrapolate to in situ conditions.

Aggregation in clusters and microbial mats is a phenomenon observed for many planktonic, benthic and terrestrial cyanobacteria. In the Baltic Sea, *Nodularia* is well-known for forming dense toxic surface scums that cause considerable nuisance along the coastlines every summer. It 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 become dominant (Kononen, 1992; Sellner, 1997; Stal et al., 2003). Hence, it appears that *Nodularia* benefits from the physical or chemical microenvironment prevailing in surface aggregations but the mechanisms involved are poorly known and 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, cyanobacteria outcompete eukaryotes under high pH and low CO_2 conditions of freshwater blooms (Shapiro and Wright, 1990; Oliver and Ganf, 2000). Observations that dispersed *Nodularia* filaments were not fertilised by elevated [CO_2], 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 could be an ecological specialisation that can not be down regulated sufficiently to benefit from [CO_2] as high as applied in the present study.

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 preference 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 a few hundreds of ppm is questionable. Considering a pH of 9 inside the aggregations, it is conceivable that a more acidic seawater 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 that must be considered when assessing future oceanic carbon and nitrogen fluxes.

The present study clearly shows that under pre-bloom conditions, with single filaments dispersed in the upper water column, cell division is negatively influenced by ocean

acidification. This could lead to a progressive delay in the formation of aggregates and thus in 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]. *Anabaena*, a cyanobacterium co-occurring with *Nodularia*, is also not fertilised by elevated [CO_2] and reacts with shifts in elemental composition different than those reported here for *Nodularia* (unpublished data). 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 (Tortell et al., 2002a). 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).

Acknowledgements. The authors thank Michael Meyerhöfer for dissolved inorganic carbon analyses, Kerstin Nachtigall for POC/PON measurements, Sebastian Krug for assistance with filtration and Manfried Dietsch for supporting the development of the cell count method.

Edited by: J.-P. Gattuso

References

Allen, M. M.: Cyanobacterial cell inclusions, Annu. Rev. Microbiol., 38, 1–25, 1984.

Arrigo, K. R.: Marine microorganisms and global nutrient cycles, Nature, 437, 349–355, 2005.

Badger, M. R., Price, G. D., Long, B. M., and Woodger, F. J.: The environmental plasticity and ecological genomics of the cyanobacterial CO_2 concentrating mechanism, J. Exp. Bot., 57, 259–265, 2005.

Barcelos e Ramos, J., Biswas, H., Schulz, K. G., La Roche, J., and Riebesell, U.: Effect of rising atmospheric carbon dioxide on the marine nitrogen fixer *Trichodesmium*, Global Biogeochem. Cy., 21, 2028, doi:2010.1029/2006GB002898, 2007.

Beardall, J. and Giordano, M.: Ecological implications of microalgal and cyanobacterial CO_2 concentrating mechanisms, and their regulation, Funct. Plant Biol., 29, 335–347, 2002.

Böhme, H.: Regulation of nitrogen fixation in heterocyst-forming cyanobacteria, Trends Plant Sci., 3, 346–351, 1998.

Breitbarth, E., Mills, M. M., Friedrichs, G., and La Roche, J.: The Bunsen gas solubility coefficient of ethylene as a function of temperature and salinity and its importance for nitrogen fixation assays, Limnol. Oceanogr.-Meth., 2, 282–288, 2004.

Burkhardt, S., Zondervan, I., and Riebesell, U.: Effect of CO_2 concentration on C:N:P ratio in marine phytoplankton: A species comparison, Limnol. Oceanogr., 44, 683–690, 1999.

Capone, D. G.: Determination of nitrogenase activity in aquatic samples using the acetylene reduction procedure, in: Handbook of methods in aquatic microbial ecology, edited by: Kemp, P. F., Sherr, B. F., Sherr, E. B., and Cole, J. J., Lewis Publishers, Boca Raton, 621–631, 1993.

Chen, Y.-B., Zehr, J. P., and Mellon, M.: Growth and nitrogen fixation of the diazotrophic filamentous nonheterocystous cyanobacterium *Trichodesmium* sp. IMS 101 in defined media: Evidence for a circadian rhythm, J. Phycol., 32, 916–923, 1996.

Decoursey, T. E.: Voltage-gated proton channels and other proton transfer pathways, Physiol. Rev., 83, 475–579, 2003.

Dickson, A. G.: An exact definition of total alkalinity and a procedure for the estimation of alkalinity and total inorganic carbon from titration data, Deep-Sea Res., 28, 609–623, 1981.

Findlay, D. L.: Response of phytoplankton communities to acidification and recovery in Killarney Park and the experimental lakes area, Ontario, AMBIO: A Journal of the Human Environment, 32, 190–195 2003.

Fridlyand, L., Kaplan, A., and Reinhold, L.: Quantitative evaluation of the role of a putative CO_2-scavenging entity in the cyanobacterial CO_2-concentrating mechanism, Biosystems, 37, 229–238, 1996.

Fu, F.-X., Mulholland, M. R., Garcia, N. S., Beck, A., Bernhardt, P. W., Warner, M. E., Sañudo-Wilhelmy, S. A., and Hutchins, D. A.: Interactions between changing pCO_2, N_2 fixation, and Fe limitation in the marine unicellular cyanobacterium *Crocosphaera*, Limnol. Oceanogr., 53, 2472–2484, 2008.

Fu, F. X., Warner, M. E., Zhang, Y. H., Feng, Y. Y., and Hutchins, D. A.: Effects of increased temperature and CO_2 on photosynthesis, growth, and elemental ratios in marine *Synechococcus* and *Prochlorococcus*, J. Phycol., 43, 485–496, 2007.

Giordano, M., Beardall, J., and Raven, J. A.: CO_2 concentrating mechanisms in algae: Mechanisms, environmental modulation, and evolution, Annu. Rev. Plant Biol., 56, 99–131, 2005.

Grasshoff, K., Ehrhardt, M., and Kremling, K.: Methods of Seawater Analysis., 2 ed., Verlag Chemie, Weinheim, Germany, 1983.

Hallingbaeck, T.: Blue-green algae and cyanophilic lichens threatened by air pollution and fertilization, Svensk Botanisk Tidskrift, 85, 87–104, 1991.

Hein, M. and Sand-Jensen, K.: CO_2 increases oceanic primary production, Nature, 388, 526–527, 1997.

Hinga, K. R.: Effects of pH on coastal marine phytoplankton, Marine Ecol.-Prog. Ser., 238, 281–300, 2002.

Hoppe, H. G.: Blue-green algae agglomeration in surface water: A microbiotope of high bacterial activity, in: Lower Organisms and their Role in the Food Web: Proceedings of the 15th European Marine Biology Symposium, Kiel, Damp 2000, edited by: Rheinheimer, G., Flügel, H., Lenz, J., and Zeitschel, B., 291–303, 1981.

Hutchins, D. A., Fu, F.-X., Zhang, Y., Warner, M. E., Feng, Y., Fortune, K., Bernhardt, P. W., and Mulholland., M. R.: CO_2 control of *Trichodesmium* N_2 fixation, photosynthesis, growth rates, and elemental ratios: Implications for past, present, and future ocean biogeochemistry, Limnol. Oceanogr., 52, 1293–1304, 2007.

Indermuehle, A., Stocker, T. F., Fischer, H., Smith, H. J., Deck, B., Mastroianni, D., Tschumi, J., Blunier, T., Meyer, R., and Stauffer, B.: Holocene carbon-cycle dynamics based on CO_2 trapped in ice at Taylor Dome, Antarctica, Nature, 398, 121–126, 1999.

Intergovernmental Panel on Climate Change (IPCC): Fourth Assessment Report, online available at: http://www.ipcc.ch, last access: February 2009, 2007.

Kangatharalingam, N., Dodds, W. K., Priscu, J. C., and Paerl., H. W.: Nitrogenase acitivity, photosynthesis, and the degree of heterocyst aggregation in the cyanobacterium *Anabaena flos-aquae*,

J. Phycol., 27, 680–686, 1991.

Kester, D. R., Duedall, I. W., Connors, D. N., and Pytkowicz, R. M.: Preparation of artificial seawater, Limnol. Oceanogr., 12, 176–179, 1967.

Keeling, R. F., Piper, S. C., Bollenbacher, A. F., and Walker, J. S.: Atmospheric Carbon Dioxide Record from Mauna Loa, Carbon Dioxide Research Group, Scripps Institution of Oceanography, University of California La Jolla, California 92093-0444, USA, online available at: http://cdiac.ornl.gov, last access: August 2009.

Koblížek, M., Komenda, J., Masojídek, J., and Pechar, L.: Cell aggregation of the cyanobacterium *Synechococcus elongatus*: Role of the electron transport chain, J. Phycol., 36, 662–668, 2000.

Kononen, K.: Dynamics of the toxic cyanobacterial blooms in the Baltic Sea, Finn. Mar. Res., 261, 3–36, 1992.

Kranz, S. A., Sültemayer, D., Richter, K., and Rost, B.: Carbon acquisition by Trichodesmium: The effect of pCO_2 and diurnal changes, Limnol. Oceanogr., 54, 548–559, 2009.

Larsson, U., Hajdu, S., Walve, J., and Elmgren, R.: Baltic Sea nitrogen fixation estimated from the summer increase in upper mixed layer total nitrogen, Limnol. Oceanogr., 46, 811–820, 2001.

Levitan, O., Rosenberg, G., Setlik, I., Setlikova, E., Griegel, J., Klepetar, J., Prasil, O., and Berman-Frank, I.: Elevated CO_2 enhances nitrogen fixation and growth in the marine cyanobacterium *Trichodesmium*, Glob. Change Biol., 13, 531–538, 2007.

Meehl, G. A., Stocker, T. F., Collins, W. D., Friedlingstein, A. T., Gaye, A. T., Gregory, J. M., Kitoh, A., Knutti, R., Murphy, J. M., Noda, A., Raper, S. C. B., Watterson, I. G., Weaver, A. J., and Zhao, Z.: Global climate projections, in: Climate Change 2007, The physical science basis, edited by: Solomon, S., Qin, D., and Manning, M., Technical support unit, IPCC working group 1, Cambridge University Press, 748–845, 2007.

Mollenhauer, D., Bengtsson R., and Lindstrom, E.-A.: Macroscopic cyanobacteria of the genus *Nostoc*: a neglected and endangered constituent of European inland aquatic biodiversity, Eur. J. Phycol., 34, 349–360, 1999.

Montesinos, M. L., Herrero, A., and Flores, E.: Amino acid transport systems required for diazotrophic growth in the cyanobacterium *Anabaena* sp. strain PCC 7120, J. Bacteriol., 177, 3150–3157, 1995.

Ohmori, K., Hirose, M., and Ohmori, M.: Function of cAMP as a mat-forming factor in the cyanobacterium *Spirulina platensis*, Plant Cell Physiol., 33, 21–25, 1992.

Oliver, R. L. and Ganf, G. G.: Freshwater blooms, in: The Ecologie of Cyanobacteria, edited by: Whitton, B. A. and Potts, M., Kluwer Academic Publishers, Dortrecht NL, 149–194, 2000.

Paerl, H. W., Bland, P. T., Bowles, N. D., and Haibach, M. E.: Adaptation to high-intensity, low-wavelength light among surface blooms of the cyanobacterium *Microcystis aeruginosa*, Appl. Environ. Microbiol., 49, 1046–1052, 1985.

Ploug, H.: Cyanobacterial surface blooms formed by *Aphanizomenon* sp. and *Nodularia spumigena* in the Baltic Sea: Small-scale fluxes, pH, and oxygen microenvironments, Limnol. Oceanogr., 53, 914–921, 2008.

Raven, J. A. and Johnston, A. M.: Mechanisms of inorganic-carbon in marine phytoplankton and their implications for the use of other resources, Limnol. Oceanogr., 36, 1701–1714, 1991.

Riebesell, U.: Effects of CO_2 enrichment on marine phytoplankton, J. Oceanogr., 60, 719–729, 2004.

Riebesell, U., Schulz, K. G., Bellerby, R. G., Botros, M., Fritsche, P., Meyerhöfer, M., Neill, C., Nondal, G., Oschlies, A., Wohlers, J., and Zöllner, E.: Enhanced biological carbon consumption in a high CO_2 ocean, Nature, 450, 545–548, 2007.

Rost, B., Zondervan, I., and Wolf-Gladrow, D.:Sensitivity of phytoplankton to future changes in ocean carbonate chemistry: current knowledge, contradictions and research directions, Mar. Ecol. Prog. Ser., 373, 227–237, 2008.

Sabine, C. L., Feely, R. A., Gruber, N., Key, R. M., Lee, K., Bullister, J. L., Wanninkhof, R., Wong, C. S., Wallace, D. W. R., Tilbrook, B., Millero, F. J., Peng, T., Kozyr, A., Ono, T., and Rios, A. F.: The oceanic sink for anthropogenic CO_2, Science, 305, 367–371, 2004.

Schneider, B., Nausch, G., Nagel, K., and Wasmund, N.: The surface water CO_2 budget for the Baltic Proper: A new way to determine nitrogen fixation, J. Marine Sy., 42, 53–64, 2003.

Schulz, K. G., Barcelos e Ramos, J., Zeebe, R. E., and Riebesell, U.: CO_2 perturbation experiments: similarities and differences between dissolved inorganic carbon and total alkalinity manipulations, Biogeosciences Discuss., 6, 4441–4462, 2009, http://www.biogeosciences-discuss.net/6/4441/2009/.

Sellner, K. G.: Physiology, ecology, and toxic properties of marine cyanobacteria blooms, Part 2: The ecology and oceanography of harmful algal blooms, Limnol. Oceanogr., 42, 1089-1104, 1997.

Shapiro, J. and Wright, D. I.: Current beliefs regarding dominance by blue- greens: The case for the importance of CO_2 and pH, Verhandlungen IVTLAP, 24, 38–54, 1990.

Sharp, J. H.: Improved analysis for "particulate" organic carbon and nitrogen from seawater, Limnol. Oceanogr., 19, 984–989, 1974.

Shibata, M., Ohkawa, H., Kaneko, T., Fukuzawa, H., Tabata, S., Kaplan, A., and Ogawa, T.: Distinct constitutive and low-CO_2-induced CO_2 uptake systems in cyanobacteria: Genes involved and their phylogenetic relationship with homologous genes in other organisms, P. Natl. Acad. Sci. USA, 98, 11789–11794, 2001.

Stal, L. J., Albertano, P., Bergman, B., Bröckel, K. v., Gallon, J. R., Hayes, P. K., Sivonen, K., and Walsby, A. E.: Baltic Sea cyanobacteria. An investigation of the structure and dynamics of water blooms of cyanobacteria in the Baltic Sea-responses to a changing environment, Con. Shelf Res., 23, 1695–1714, 2003.

Stoll, M. H. C., Bakker, K., Nobbe, G. H., and Haese, R. R.: Continuous-flow analysis of dissolved inorganic carbon in sea water, Anal. Chem., 73, 4111–4116, 2001.

Surosz, W., Palinska, K. A., and Rutkowska, A.: Production of transparent exopolymer particles (TEP) in the nitrogen fixing cyanobacterium *Anabaena flos-aque*, Oceanologia, 48, 385–394, 2006.

Thomas, H. and Schneider, B.: The seasonal cycle of carbon dioxide in Baltic Sea surface water, J. Marine Sy., 22, 53–67, 1999.

Tortell, P. D., DiTullio, G. R., Sigman, D. M., and Morel F. M. M.: CO_2 effects on taxonomic composition and nutrient utilization in an Equatorial Pacific phytoplankton assemblage Mar. Ecol.-Prog. Ser., 236, 37–43, 2002a.

Tortell, P. D. and Morel, F. M. M.: Sources of inorganic carbon for phytoplankton in the eastern Subtropical and Equatorial Pacific Ocean, Limnol Oceanogr., 47, 1012–1022, 2002b.

Welschmeyer, N. A.: Fluorometric analysis of chlorophyll a in the presence of chlorophyll b and pheopigments, Limnol. Oceanogr., 29, 1985–1992, 1994.

Whitton, B. A. and Potts, M.: Introduction to the Cyanobacteria, in: The Ecologie of Cyanobacteria, edited by: Whitton, B. A. and Potts, M., Kluwer Academic Publishers, Dortrecht NL, 1–11, 2000.

Wolk, C. P., Ernst, A., and Elhai, J.: Heterocyst metabolism and development, in: The Mollecular Biologie of Cyanobacteria, edited by: Bryant, D. A., Kluwer Academic Publishers, 769–823, 1994.

Yang, Y. and Gao, K.: Effects of CO_2 concentrations on the freshwater microalgae, *Chlamydomonas reinhardtii*, *Chlorella pyrenoidosa* and *Scenedesmus obliquus (Chlorophyta)*, J. Appl. Phycol., 15, 379–389, 2003.