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# Influence of elevated CO<sub>2</sub> 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<sub>2</sub> emitted to the atmosphere from human activities. As this CO<sub>2</sub> 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<sub>2</sub> at the expense of CO<sub>3</sub><sup>2-</sup> concentrations. While the decrease in [CO<sub>3</sub><sup>2-</sup>] and/or increase in [H<sup>+</sup>] has been found to adversely affect many calcifying organisms, some photosynthetic organisms appear to benefit from increasing [CO<sub>2</sub>]. 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 *p*CO<sub>2</sub>. With the mechanism underlying this CO<sub>2</sub> 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<sub>2</sub>-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<sub>2</sub> concentrations to rise from an interglacial level of 280 ppm in preindustrial times, (Indermuhle et al., 1999) to presently 380 ppm (Keeling and Whorf, 2005). In the case of unabated CO<sub>2</sub> 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<sub>2</sub> emitted since the beginning of the industrialisation (Sabine et al., 2004). Due to the reaction of CO<sub>2</sub> 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<sub>2</sub> emission scenario (IPCC IS92a; Meehl et al., 2007).

As photosynthetic CO<sub>2</sub> fixation is substrate limited under current atmospheric CO<sub>2</sub>/O<sub>2</sub> ratios all photoautotrophic organisms evolved active carbon concentrating mechanisms (CCM), providing elevated [CO<sub>2</sub>] at the site of carboxylation. In seawater, CO<sub>2</sub> concentration represents less than 1% of the inorganic carbon species and does indeed limit photosynthetic carbon fixation rates (Giordano et al., 2005). Elevated CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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 *Crocospaera* (Fu et al., 2008). A combined positive effect of CO<sub>2</sub> 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-

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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<sub>2</sub> related shifts in elemental ratios were also found in the studies on *Trichodesmium*, *Crocospaera* 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<sub>2</sub>. 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<sub>2</sub> 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<sub>2</sub> and CO<sub>3</sub><sup>2-</sup> concentrations (Thomas and Schneider, 1999).

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In this study, aggregation is avoided as microclimate effects in the aggregates concerning the CO<sub>2</sub>/O<sub>2</sub> conditions might cause significant alteration of CO<sub>2</sub> related physiological effects (Ploug, 2008). Carbon and nitrogen fixation of *Nodularia spumigena* were investigated under five different CO<sub>2</sub> concentrations. Simulated CO<sub>2</sub> 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<sub>2</sub>] 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<sup>-2</sup> s<sup>-1</sup> 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 homogeneously 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<sub>2</sub> treatments, pre-cultures were grown for 13 days (for replicates 1–9), respectively 18 days for the two high CO<sub>2</sub>-concentration treatments (replicates 11–15) to reach similar cell densities at the start of the experiment.

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Pre-culture incubation corresponded to about seven to eight cell generations. The experiment was started with an initial Chl-*a* concentration of 1 μg l<sup>-1</sup> 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 μmol l<sup>-1</sup>). 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 μmol kg<sup>-1</sup>). 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> value of 150 ppm was chosen because at the time of *Nodularia* bloom development, i.e. early to mid summer, pCO<sub>2</sub> levels in the Gotland Sea are typically between 100 and 200 ppm (Thomas and Schneider, 1999). These comparatively low CO<sub>2</sub> levels result from intense biological activity earlier in the season combined with a low buffer capacity of the Baltic Sea brackish waters.

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## 2.3 Measuring methods

### 2.3.1 Seawater carbonate system

DIC was measured after Stoll et al. (2001) in a QUAATRO analyzer (Bran and L bbecke GmbH, Norderstedt, Germany) equipped with a XY-2 sampling unit. The precision and accuracy of this method are  $\sim 2\text{--}3\ \mu\text{mol kg}^{-1}$ . 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  $\sim 2\ \mu\text{mol kg}^{-1}$ . 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<sub>2</sub> measurement (Marine Physical Laboratory, University of California, A. G. Dickson).  $p\text{CO}_2$  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  $\sim 200$  mbar on glass fibre filters (Whatman GF/F 25 mm  $\varnothing$ ) and stored frozen at  $-18^\circ\text{C}$ . 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  $\sim 200$  mbar on a

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combusted filter (Whatman GF/F 25 mm  $\varnothing$ ) and subsequently stored at  $-18^\circ\text{C}$ . 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  $\sim 200$  mbar on combusted filters (Whatman GF/F 25 mm  $\varnothing$ ) and subsequently stored at  $-18^\circ\text{C}$ . 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  $\sim \pm 0.2\ \mu\text{mol l}^{-1}$ .

### 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 ( $\sim 25\%$  of the headspace volume). After four hours of incubation at a light intensity of  $85\ \mu\text{mol photon m}^{-2}\ \text{s}^{-1}$ , 300  $\mu\text{l}$  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  $\varnothing$  1.2  $\mu\text{m}$  pore size AE95 Schleicher and Sch ll, 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

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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 Manfred 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 ( $\mu$ ) 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  $\sim 0.52 \text{ d}^{-1}$  at the lowest  $\text{CO}_2$  level and the minimum values of  $\sim 0.33 \text{ d}^{-1}$  at

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elevated  $\text{CO}_2$  levels (Fig. 1a). This resulted in a total decrease in cell division rate of 36% over the experimental  $\text{CO}_2$  range. A slight decrease in cellular nitrogen fixation in relation to rising  $p\text{CO}_2$  was tested to be barely significant ( $p=0.014$ ) (Fig. 1c). A significant increase in cellular carbon and phosphorus content with rising  $[\text{CO}_2]$  was detected (Fig. 2a, c). Taking the regression line as a mean, carbon and phosphorus cell quota increased from low to high  $\text{CO}_2$  treatments by 32% and 30%, respectively, while cellular Chlorophyll-*a* did not change with  $\text{CO}_2$  treatment (Fig. 1b). In contrast to carbon and phosphorus cell quota, the cellular nitrogen content did not show a clear trend with  $\text{CO}_2$  (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  $\text{CO}_2$  range (Fig. 3a, c). A decreasing trend with  $\text{CO}_2$  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  $\text{CO}_2$  treatment. The mean length of vegetative cells was  $3.8 \pm 0.46 \mu\text{m}$  ( $n=601$ ), at a filament width of  $10.7 \pm 0.91 \mu\text{m}$  ( $n=125$ ). Heterocyst length was  $8.8 \pm 1.2 \mu\text{m}$  ( $n=63$ ). In all samples, one of 12 cells  $\pm 1$  was a heterocyst.

Carbon to nitrogen ratios exhibited a highly significant ( $p<0.001$ ) increase (26%) in response to elevated  $[\text{CO}_2]$  and lowered pH. At low  $[\text{CO}_2]$ , C/N was about 5.5 and thus below the Redfield ratio while a maximum value (7.0) at high  $[\text{CO}_2]$  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  $[\text{CO}_2]$ , 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  $\text{CO}_2$ , complementary to C/N ratio (Fig. 4c).

#### 4 Discussion

Various studies in recent years have demonstrated direct effects of rising CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>. Aside from the unexpected slope of this trend, a surprising observation also was that the inverse relationship of cell division rate and pCO<sub>2</sub> extended to a CO<sub>2</sub> 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<sub>2</sub> concentrations suggests that maximum cell division rate may occur at even higher pH and lower [CO<sub>2</sub>] 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<sub>2</sub> 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<sub>2</sub>SO<sub>4</sub>, HNO<sub>3</sub>) 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

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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<sub>2</sub>], 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<sub>2</sub> 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<sub>2</sub>] 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<sub>2</sub> 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

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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

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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 *Anabaena 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

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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<sub>2</sub>. 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<sub>2</sub> conditions of freshwater blooms. Observations that dispersed *Nodularia* filaments showed no CO<sub>2</sub> 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<sub>2</sub> limited microenvironment of a dense cyanobacterial bloom is possibly an ecological specialisation that can not be down regulated sufficiently to profit from [CO<sub>2</sub>] 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<sub>2</sub>] 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

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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<sub>2</sub> 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<sub>2</sub>]. For *Anabaena*, a cyanobacterium often found together with *Nodularia*, there is indication that there will be a different reaction in response to rising [CO<sub>2</sub>] (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<sub>2</sub> 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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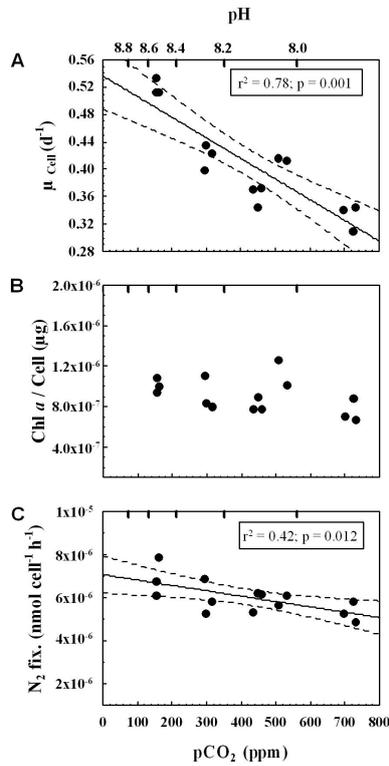
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**Table 1.** Values of the carbonate system in the 14 experimental units. Alkalinity was measured in the end of the experiment while DIC was determined at the beginning and in the end. pH values (on the free scale) and CO<sub>2</sub> partial pressures are calculated for the point at which half of the DIC consumed during the experiment has been taken up.

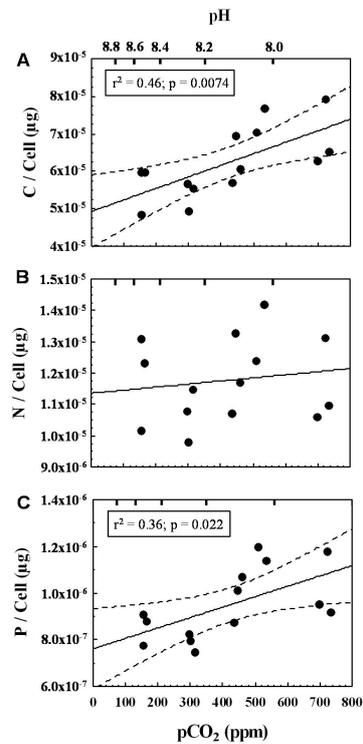
Replicate	$p\text{CO}_2$ (ppm)	pH	Alkalinity ( $\mu\text{mol kg}^{-1}$ )	DIC Start ( $\mu\text{mol kg}^{-1}$ )	DIC End ( $\mu\text{mol kg}^{-1}$ )
1	162	8.55	2188	1981	1946
2	153	8.57	2193	1981	1933
3	154	8.57	2191	1981	1933
4	297	8.31	2090	1981	1943
5	295	8.31	2091	1981	1945
6	313	8.29	2088	1981	1950
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

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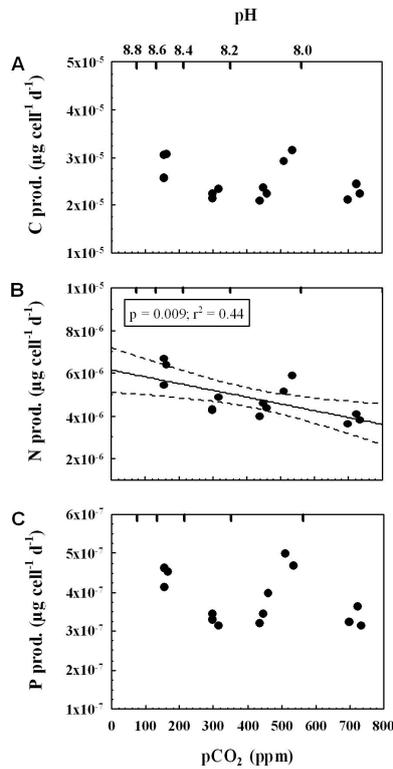
**Fig. 1.** Cellular division rate (a), cellular chlorophyll-*a* content (b) and nitrogen fixation rate (c) as a function of CO<sub>2</sub> partial pressure and corresponding pH. Each data point represents one bottle. For the regression line (solid) regression coefficient  $r^2$  and  $p$  value are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

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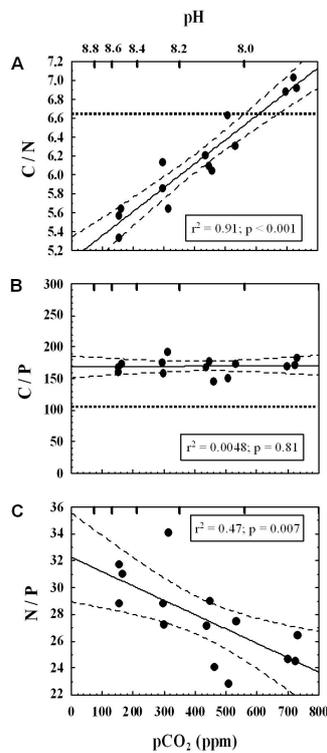
**Fig. 2.** Variations in cell quota of carbon (a), nitrogen (b) and phosphorus (c) as a function of CO<sub>2</sub> partial pressure and corresponding pH. Each data point represents results from one bottle. For the regression line (solid) regression coefficient  $r^2$  and  $p$  value are given in the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

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**Fig. 3.** Cellular production rates of POC (a), PON (b) and POP (c) as a function of CO<sub>2</sub> 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%.

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**Fig. 4.** Variation in C/N (a), C/P (b) and N/P (c) molar ratios as a function of CO<sub>2</sub> 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%.

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