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Response of *Nodularia spumigena* to pCO$_2$ – Part 2: Exudation and extracellular enzyme activities

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Abstract. The filamentous and diazotrophic cyanobacterium *Nodularia spumigena* plays a major role in the productivity of the Baltic Sea as it forms extensive blooms regularly. Under phosphorus limiting conditions *Nodularia spumigena* have a high enzyme affinity for dissolved organic phosphorus (DOP) by production and release of alkaline phosphatase. Additionally, they are able to degrade proteinaceous compounds by expressing the extracellular enzyme leucine aminopeptidase. As atmospheric CO$_2$ concentrations are increasing, we expect marine phytoplankton to experience changes in several environmental parameters, including pH, temperature, and nutrient availability. The aim of this study was to investigate the combined effect of CO$_2$-induced changes in seawater carbonate chemistry and of phosphate deficiency on the exudation of organic matter, and its subsequent recycling by extracellular enzymes in a *Nodularia spumigena* culture. Batch cultures of *Nodularia spumigena* were grown for 15 days under aeration with low (180 µatm), medium (380 µatm), and high (780 µatm) CO$_2$ concentrations. Obtained pCO$_2$ levels in the treatments were on median 315, 353, and 548 µatm CO$_2$, respectively. Extracellular enzyme activities as well as changes in organic and inorganic compound concentrations were monitored. CO$_2$ treatment–related effects were identified for cyanobacterial growth, which in turn influenced the concentration of mucinous substances and the recycling of organic matter by extracellular enzymes. Biomass production was increased by 56.5 % and 90.7 % in the medium and high pCO$_2$ treatment, respectively, compared to the low pCO$_2$ treatment. In total,

significantly more mucinous substances accumulated in the high pCO$_2$ treatment, reaching 363 µg Xeq L^{-1} compared to 269 µg Xeq L^{-1} in the low pCO$_2$ treatment. However, cell-specific rates did not change. After phosphate depletion, the acquisition of P from DOP by alkaline phosphatase was significantly enhanced. Alkaline phosphatase activities were increased by factor 1.64 and 2.25, respectively, in the medium and high compared to the low pCO$_2$ treatment. We hypothesise from our results that *Nodularia spumigena* can grow faster under elevated pCO$_2$ by enhancing the recycling of organic matter to acquire nutrients.

1 Introduction

Cyanobacteria play an important role in the productivity of the Baltic Sea and form regularly extensive blooms (Finni et al., 2001). One of the typical bloom-forming species is the filamentous *Nodularia spumigena* (Sellner, 1997), which has the physiological capacity to fix atmospheric nitrogen. The estimated annual amount of fixed nitrogen in the Baltic Sea by cyanobacteria is 180–430 Gg N and equal to the total nitrogen input from rivers (480 Gg N yr^{-1}) and atmospheric deposition ($\sim$ 200 Gg N yr^{-1}) (Larsson et al., 2001; Schneider et al., 2003). Therefore, *Nodularia* is highly important for ecosystem functions in the Baltic Sea.

Nutrient concentrations, especially nitrogen and phosphorus, are the potential limiting factors for phytoplankton growth in the ocean, with phosphorus being suggested as the

more important nutrient for long-term productivity (Tyrell, 1999). As inorganic phosphorus is depleted rapidly, a potential alternative for phytoplankton growth is the utilisation of dissolved organic phosphorus (DOP) (Björkman and Karl, 1994; Nausch, 1998a; Nausch and Nausch, 2006). Its availability may be an important factor influencing the distribution of diazotrophic cyanobacteria in the Baltic Sea (Niemi, 1979) as phosphate bioavailability controls the ability to fix N_2 (Paytan and McLaughlin, 2007). In the Baltic Sea, the DOP fraction in surface waters ranges between 0.20 to 0.23 µM in the central basin (Nausch et al., 2004) and 0.50 to 0.90 µM in the Gulf of Riga (Põder et al., 2003), and can constitute as much as 70–100 % of total phosphorus (Kononen et al., 1992; Nausch et al., 2004). This DOP pool is estimated to support 20 % (range of 12–30 %) of the phytoplankton production in the field (Mather et al., 2008). Hence, the ability to use this source efficiently is of great ecological importance for diazotrophs during nutrient limitation (Degerholm et al., 2006; Paytan and McLaughlin, 2007). *Nodularia* and similar species appear well adapted to the stratified and P-limited surface waters, which are predominantly found in these waters of the Baltic Sea in summer (Degerholm et al., 2006).

Gel particles play an important role in aggregation and export of organic and inorganic matter (Logan et al., 1995; Passow et al., 2001; Shipe et al., 2002; Engel et al., 2004b). These particles, consisting mainly of acidic sugars, are called transparent exopolymer particles (TEP) and can be visualised under the microscope by Alcian Blue staining. Proteinaceous particles can be stained with Coomassie Blue and are therefore referred to as Coomassie stainable particles (CSP). Both TEP and CSP are potential food sources for bacteria but also substrate to grow upon. One origin of these exopolymers in the sea is exudation of carbon compounds by phytoplankton under nutrient limitation (Passow, 2002; Engel et al., 2002a, 2004a). Relatively little is known about exudation and gel particle formation in cyanobacteria communities (Engel et al., 2002b; Engel, 2002). Changes in light intensity were shown to have an increasing effect on exudation of DON and DOC in cultures of *Nodularia spumigena*. Up to 89 % of the fixed nitrogen and 53 % of the fixed carbon were released during the light period (Wannicke et al., 2009). Besides exudation, various species of unicellular and filamentous cyanobacteria produce large amounts of extracellular polymeric substances consisting predominantly of polysaccharides (Otero and Vincenzini, 2004), which form a mucus layer around the cell. The function of this mucus layer and factors regulating the production, however, are not completely understood yet (Nausch, 1996; Otero and Vincenzini, 2004).

Microbes consume carbohydrates and proteins to meet their energy and nutrient requirements and to build up biomass. Therefore, they hydrolyse high molecular weight dissolved organic matter (DOM) with extracellular enzymes (Chrost et al., 1989). Highest activity rates and affinity of extracellular enzymes are usually measured towards the end of a phytoplankton bloom (Chrost et al., 1989). While α- and β-glucosidase and aminopeptidase activity were typically found to be associated with the bacterial fraction, alkaline phosphatase activity was associated also with phytoplankton (Chrost et al., 1989). Recent studies showed that cultured, axenic strains of *Nodularia spumigena* also seem to be able to express leucine aminopeptidase to degrade proteins (Stoecker et al., 2005).

The alkaline phosphatase catalyses the hydrolytic breakdown of the PO_4^{3-} end from DOM and is expressed in response to phosphate limitation in many bacterial and phytoplankton species (Azam et al., 1983; Chrost and Overbeck, 1987; Beardall et al., 2001; Labry et al., 2005). In the Baltic Sea, alkaline phosphatase activity seems to be directly related to the abundance of heterocystous cyanobacteria (Nausch et al., 2004). Increasing phosphatase activities were determined with decreasing phosphate concentrations during *Nodularia* blooms (Huber and Hamel, 1985a, b). Cultures of *Trichodesmium* IMS101 can grow with DOP as the only source of phosphorus, suggesting that DOP should be included in estimates of P availability in surface waters (Mulholland et al., 2002).

Due to rising atmospheric $p\text{CO}_2$ the ocean pH has been decreasing constantly since the beginning of the Anthropocene – around 1800 (Zalasiewicz et al., 2008). By the year 2100, a further decrease by 0.3 units from current conditions is expected (Wolf-Gladrow et al., 1999; Feely et al., 2010). The atmospheric CO_2 concentrations have increased since glacial periods from 180 µatm to 380 µatm nowadays and we expect future values to climb to around 780 µatm by the year 2100 (Parry et al., 2007; Meehl et al., 2007; Raupach et al., 2007). The effect of ocean acidification on marine microbes and the turnover of organic matter are scarcely explored (Joint et al., 2010). During the past decades, an increase in the frequency (Sellner et al., 2003) and extension (Kahru et al., 1994) of cyanobacteria blooms has been detected and mainly attributed to global warming and eutrophication (O'Neil et al., 2012). Considering the fact that marine microbes already experience high variations in CO_2 and pH, they might have the flexibility to accommodate pH change and therefore the microbial-driven biogeochemical processes will probably not change dramatically (Joint et al., 2010). On the other hand, it has been shown that pH has a regulating effect on enzyme activities. Some hydrolytic enzymes have been shown to have their optimum at a pH below present seawater pH. Piontek et al. (2010) showed that a decrease in seawater pH as expected for the near future increases enzymatic hydrolysis rates of polysaccharides and accelerates the bacterial degradation of organic carbon. During a mesocosm study aminopeptidase activity was significantly higher at elevated $p\text{CO}_2$ (Grossart et al., 2006).

A direct coupling between inorganic carbon acquisition and organic carbon exudation was observed during incubation experiments in the central Baltic Sea (Engel, 2002). Future increase in atmospheric CO_2 may, however, not

necessarily lead to a higher exudation rate as the latter seemed to be already at its maximum under present CO$_2$ concentration in some ecosystems (Engel, 2002). Nevertheless, a mixed mesocosm phytoplankton community treated with high CO$_2$ showed significantly enhanced TEP production normalised to cell abundance (Engel et al., 2004a). Increasing pCO$_2$ may potentially affect N$_2$ fixation and increase the release of DOM but also the recycling of nutrients by *Nodularia*, and may therefore have consequences for nutrient cycling and the export of organic matter in the Baltic Sea. So far, there are only two studies published on the effects of increasing CO$_2$ on *Nodularia* spp. (Czerny et al., 2009; Wannicke et al., 2012). Yet observations on changes in extracellular enzyme activities and turnover of organic matter in cyanobacteria blooms due to ocean acidification are still lacking.

This is the second of three companion papers on a major culture experiment with *Nodularia spumigena* (Wannicke et al, 2012; Unger et al., 2012). The aim of this study was to examine the relationship between pCO$_2$ and diazotrophic growth of *Nodularia spumigena* and the related fluxes of carbon, nitrogen, and phosphorus. In particular we wanted to investigate the effect of ocean acidification on the exudation of organic matter and its subsequent recycling by extracellular enzymes in a *Nodularia spumigena* batch experiment. Enzyme activities as well as changes in organic and inorganic compound concentrations were measured over 15 days of incubation at three different pCO$_2$ levels.

2 Material and methods

2.1 Cultivation and experimental setup

Details of the experimental setup and culture conditions can be found in Wannicke et al. (2012). Axenic precultures of the cyanobacterium *Nodularia spumigena* were grown in F/2 medium in batch bottles at 15 °C with a light cycle of 16 : 8 (cool, white fluorescent lighting, 100 µmol photons m^{-2} s^{-1}). The precultures were aerated for three days with premixed gases (Linde gas) of 180, 380, and 780 ppm CO$_2$, representing past, present, and future atmospheric pCO$_2$, respectively. The premixed gas was filtered through seawater to increase humidity of the air to avoid water evaporation from the batch bottles. During the duration of the experiment the batch cultures were aerated for one hour per day. Aeration was not sufficient to yield the target pCO$_2$ concentrations (Wannicke et al., 2012), and carbonate chemistry may have been altered additionally by cellular carbon uptake. However, the three obtained pCO$_2$ levels differed from each other and therefore we refer to them as "low", "medium" and "high" pCO$_2$ treatments. The low pCO$_2$ treatment ranged from 249 to 499 µatm CO$_2$ with a median of 315 µatm. The medium pCO$_2$ treatment reached 287 to 571 µatm with a median of 353 µatm. The high pCO$_2$

level was 395 to 630 µatm with a median of 548 µatm (Wannicke et al., 2012).

At the beginning of the experiment (day 0), 36 bottles with nutrient-depleted (below detection limit), four-month aged and sterile-filtered Baltic Sea water were inoculated with *N. spumigena* to a starting concentration of 0.8 µg chl a L^{-1}. To stimulate growth, 0.35 µM PO$_4^{3-}$ were added at day 0 and on day 3 as phosphate was already depleted in the cultures. Three bottles with sterile seawater remained as a blank to determine background concentrations of inorganic and organic compounds in the seawater. The bottles were aerated with premixed gases of the three pCO$_2$ levels, resulting in 12 replicates of each CO$_2$ treatment. We followed the build-up and decline of the phytoplankton blooms over a 15-day period, with sampling on days 0, 3, 9 and 15. Three replicate bottles of each CO$_2$ treatment were harvested at each sampling point. In the following we refer to the average concentration of the three replicates and its standard deviation for all parameters. The experiment was conducted at 23 °C and the day-to-night cycle was adjusted to 16:8 h with a light intensity of 200 µmol photons m^{-2} s^{-1}.

2.2 Carbonate chemistry

The carbonate chemistry was determined by measuring pH and dissolved inorganic carbon (DIC) on each sampling day. The pH was measured using an electrode (Knick Mikroprozessor pH Meter 761 with Type SE 100 glass electrode), repeatedly calibrated with NBS buffer. Values of pH are given relative to total scale. DIC was analysed according to Johnson et al. (1993) directly after sampling using the colorimetric SOMMA system. We used carbon reference material provided by A. Dickson (University of California, San Diego). Analysis precision was ±2 µmol kg^{-1}. pCO$_2$ and total alkalinity (TA) were calculated with CO2SYS (Lewis et al., 1998).

2.3 Analysis of inorganic and organic compounds

Determination of inorganic nutrients (NH$_4^+$, NO$_3^-$ and PO$_4^{3-}$) was accomplished by filtering 60 mL sample through a combusted GF/F filter and measuring colorimetrically in a spectrophotometer U 2000 (Hitachi-Europe GmbH, Krefeld, Germany) according to the method of Grasshoff et al. (1983). Ammonium concentrations remained undetectable throughout the course of the experiment.

To measure the chlorophyll a content (Chl a), 100 mL water samples were filtered onto Whatman GF/F filters and the filters were stored in liquid nitrogen or at −80 °C. After thawing, they were extracted with 96 % ethanol for at least 3 h and the Chl a fluorescence was measured with a TURNER fluorometer (10-AU-005) at an excitation wavelength of 450 nm and an emission of 670 nm (HELCOM, 2005). The chlorophyll concentration was calculated according to the method of Jeffrey and Welschmeyer (1997).

Samples for dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) analysis were filtered through combusted GF/F filters, collected in 20 mL combusted (8 h, 500 °C) glass ampoules, acidified with 80 μL of 85 % phosphoric acid and stored at 0–2 °C for 10 months. DOC and TDN concentrations were determined simultaneously in the filtrate by high temperature catalytic oxidation with a Shimadzu TOC-VCSH analyser equipped with a Shimadzu TNM-1 module. DOC and TDN concentrations were average values of quadruplicate measurements. Values of TDN were corrected for nitrate, and ammonium, and thereafter referred to as DON.

For the determination of total phosphorus (TP), 40 mL unfiltered samples were taken and stored at −20 °C up to two months. For measuring dissolved phosphorus (DP), 40 mL samples were filtered through combusted (450 °C, 4 h) Whatman GF/F filters. The thawed samples were oxidised with an alkaline peroxodisulfate solution (Grasshoff et al., 1983) in a microwave (μPrep-A) to convert organic phosphorus into dissolved inorganic phosphorous (PO_4^{3-}). The PO_4^{3-} concentration was determined photometrically ($\lambda = 885$ nm) in a 10 cm cuvette. The detection limit was 0.01 μM. Dissolved organic phosphorus (DOP) was calculated by subtracting PO_4^{3-} from DP. Particulate organic phosphorus (POP) was calculated by subtracting DP from TP.

For analysis of particulate organic carbon (POC) and nitrogen (PON) concentration, 200 mL were filtered onto GF/F filters and stored at −20 °C. Concentrations were measured by means of flash combustion in a Carlo Erba EA 1108 at 1020 °C and a Thermo Finnigan Delta S mass spectrometer.

2.4 *Nodularia* filament and bacteria cell counts

Fifty mL samples were fixed with acetic Lugol's (KI/I2) solution (1 % v/v final concentration). Within the subsequent four weeks, abundance, cell length and width of *Nodularia* sp. filaments were determined with an inverted Leica microscope at 100× magnification (Utermöhl, 1958). For bacterial cell counts, 4 mL samples were preserved with 100 μL formaldehyde (1 % v/v final concentration), shock frozen in liquid nitrogen and stored at −70 °C for three months until measurement. A stock solution of SYBR GREEN (Molecular Probes) was prepared by mixing 1 μL dye with 49 μL dimethyl sulfoxide (DMSO, Sigma Aldrich, 1 : 16 diluted). A 3 μL potassium citrate solution, 10 μL of the dye stock solution and 10 μL fluoresbrite microspheres (Polysciences) were added to 300 μL of the thawed sample and incubated for 30 min in the dark. These samples were then analysed using a flow cytometer (Facs Calibur, Becton Dickinson) following the method of Gasol and del Giorgio (2000) at medium flow rate. Calculations were done using the software program Cell Quest Pro.

2.5 Transparent exopolymer particles (TEP) and Coomassie stainable particles (CSP)

Alcian Blue stains the acidic mucopolysaccharide layers surrounding cells (mucus) as well as free aggregates of acidic sugars, so-called transparent exopolymer particles (TEP). As we cannot distinguish between these two with this method, we refer to them as "mucinous substances" (Leppard, 1995). For analysis, 15 mL samples were filtered onto 0.4 μm Nuclepore filters stained with 1 mL of a calibrated Alcian Blue solution and rinsed with several mL of ultrapure water. All samples were filtered in triplicate. Before use, the staining solution was filtered (0.2 μm) to avoid particles in the dye solution. The filters were stored at −20 °C for 2–6 weeks until spectrophotometrical analysis. The amount of Alcian Blue adsorption per sample was determined colorimetrically according to Passow and Alldredge (1995). Each filter was incubated for 3 h with 6 mL of 80 % H$_2$SO$_4$ in order to dissolve the particles, and then the solution was measured at 787 nm with an UV-Vis spectrophotometer (Shimadzu UV-1700 PharmaSpec). The total concentration of mucinous substances is given in xanthan gum equivalent (Xeq), as xanthan gum was used for calibration.

For microscopic analysis of mucinous substances size and abundance, 5 mL samples were filtered in duplicate onto 0.4 μm Nuclepore filters, stained and incubated with 1 mL of Alcian Blue for 3 s, then rinsed with distilled water. Samples for the proteinaceous gel particles (CSP) were processed identically except that they were stained and incubated with 1 mL of Coomassie Blue solution for 30 s. CSP may play an important role as organic N source for heterotrophic organisms. They were barely stained in the mucus surface coating of *Nodularia* so CSP could be considered as discrete particles during this experiment. The filters were stored on cytoclear slides at −20 °C until microscopic analysis (Engel, 2009). Slides were analysed with a light microscope connected to a color video camera with 400× magnification. About 2 × 30–35 frames per slide were taken in a cross section. Particles were counted and sized semi-automatically using the software Image J (Rasband, 1997–2011). CSP size can be compared by calculating the equivalent spherical diameter (ESD) of each particle. Total mucinous substance concentration determined by Xeq was significantly related to total area of mucinous substances determined by microscopic analysis ($n = 37$, $R^2 = 0.73$, $p < 0.001$).

2.6 Extracellular enzyme activities

Fluorogenic model substrates are used to quantify potential in situ extracellular enzyme activities (Hoppe et al., 2002). The activity of extracellular enzymes (alkaline phosphatase, α- and β-glucosidase, and leucine-aminopeptidase) was determined by using 4-methylumbelliferyl (MUF)-phosphate, 4-MUF-α-glucopyranoside, 4-MUF-β-glucopyranoside, and L-leucine-4-methyl-7-coumarinylamide (MCA), respectively

(Hoppe, 1983). The fluorescent substrate analogues were added to subsamples of 180 µL volume and incubated in duplicates for 3.5–4.5 h in the dark at 25 °C. Seven different substrate concentrations ranging from 0 to 150 µM (0, 1, 5, 10, 20, 50 and 150 µM) were tested. Sample fluorescence was measured in microtiter plates with a fluorometer (FLUOstar OPTIMA, BMG Labtech, excitation 355 nm, emission 460 nm). Calibration was carried out with solutions of MUF and MCA. Detection limit for the fluorescent dye was 25 nM for MUF and 10 nM for MCA. To eliminate background fluorescence effects and to ensure that there was no significant substrate hydrolysis due to abiotic processes, control samples for every concentration of substrate added were measured with distilled water or sterilised seawater. In order to consider pH effects on the fluorescence intensity of MUF, standard solutions were adjusted to pH 7.88, 7.99, 8.0, 8.35, and 8.66. V_{max} is the maximum rate achieved by the system at saturating substrate concentrations. The activity of extracellular enzymes was calculated as the maximum hydrolysis rate (V_{max}) using the software SigmaPlot 12.0. The Michaelis–Menten constant K_m is the substrate concentration at which the reaction rate is half of V_{max}.

2.7 Data analysis and statistics

Calculations, statistical tests and illustration of the data were performed with the software packages Microsoft Office Excel 2003 and Sigma Plot 12.0 (Systat). Values given are the average of three replicates. To compare different pCO$_2$ treatments, an independent t-test was used. The significance level for all tests was $p < 0.05$. Data were tested for normality using the Shapiro–Wilk test. The relation between the organic parameters, nitrogen fixation rate (actual rates are presented in Wannicke et al., 2012) and enzyme activities was assessed by the Spearman Rank Order Correlation. For pairs with $R^2 > 0.5$, correlation coefficient $|R| > 0.7$ and p-values below 0.05, there is a strong significant relationship (**) between the two variables. For pairs with correlation coefficient $|R|$ between 0.3 and 0.7 and p-values below 0.05, there is a weak significant relationship (*) between the two variables. The pairs of variables with positive correlation coefficients R tend to increase together. For the pairs with negative correlation coefficients R, one variable tends to decrease while the other increases. There was one outlier in the data set (Sample 180-II on day 9), which contained double amount of PO$_4^{3-}$ at the beginning. It was removed to ensure equal starting conditions of the replicates.

3 Results

3.1 Biomass production

Detailed information on general bloom development, nitrogen fixation rates and cell productivity is given in Wannicke et al. (2012) and will only briefly be summarised here. *Nodu-laria* filament abundance, Chl *a* concentration, as well as POC concentrations increased over time in all treatments until day 9, which was supposed to be around the maximum of the bloom. The Chl *a* concentration rose from 0.8 µg Chl *a* L^{-1} on day 0 up to 4.6 µg L^{-1} in the low and 7.3 µg L^{-1} in the high$p$CO$_2$ treatment on day 9 (Fig. 1). We refer to this period as the "growth phase". From day 9 to day 15, Chl *a* concentrations decreased to 1.5 µg at low and 4.4 µg at high pCO$_2$, respectively. In general, Chl *a* concentrations increased with pCO$_2$. POC concentrations increased by 81.9 µmol C L^{-1} in the low, 128.1 µmol C L^{-1} in the medium and 156.2 µmol C L^{-1} in the high pCO$_2$ treatment (Fig. 2). Thus, POC increase was significantly elevated by 56.5 % and 90.7 % at medium ($p = 0,005$) and high ($p = 0.029$) pCO$_2$ treatments, respectively, compared to the low pCO$_2$ treatment. Specific growth rates (µ) per day were calculated based on changes in filament abundance, chlorophyll *a* (Chl *a*), particulate organic nitrogen (PON) and particulate organic carbon (POC) for the three pCO$_2$ treatments from day 0 to day 9. Compiled growth rates based on all parameters, presented in Wannicke et al. (2012), were significantly different between the pCO$_2$ treatments ($p < 0.05$ and $p = 0.001$). Based on POC data, growth rates were 0.0980 ± 0.0480 d^{-1} in the low, 0.1500 ± 0.0070 d^{-1} in the medium, and 0.2060 ± 0.0200 d^{-1} in the high pCO$_2$ treatments.

Heterotrophic bacteria cells counts at the start of the experiment were below the blank value (Wannicke et al., 2012). No significant growth of heterotrophic bacteria was observed over time. Bacterial cell numbers on average were $4.5 \pm 2.3 \times 10^5$ cells L^{-1} in the low, $2.4 \pm 2.1 \times 10^5$ cells L^{-1} in the medium and $4.8 \pm 2.8 \times 10^5$ cells L^{-1} in the high pCO$_2$ treatment (Wannicke et al., 2012, Table 2). Standard deviations were relatively high and cell numbers varied between replicates and over time, probably due to methodological constraints, but no systematic increase was observed over time. If some bacteria were attached to each other, to *Nodularia* or to gel particles, bacterial abundance determined by flow cytometry might have been underestimated. On the other hand, non-viable bacteria might have been included in the enumeration since also extracellular nucleic acids and dead, DNA-containing cells stain with the dye SYPBR green that is used for flow cytometry, as discussed in Wannicke et al. (2012).

3.2 Exudation and formation of gel particles

DOC concentrations were 303 ± 26 µmol L^{-1} in the low, 309 ± 21 µmol L^{-1} in the medium, and 313 ± 36 µmol L^{-1} in the high pCO$_2$ treatment. The increase in DOC during the growth phase (day 0 to day 9) in all treatments ranged between 2 and 59 µmol DOC L^{-1}. Differences between the single pCO$_2$ treatments were not significant and differences between replicates were higher than between treatments (Fig. 3). The average DON concentration was
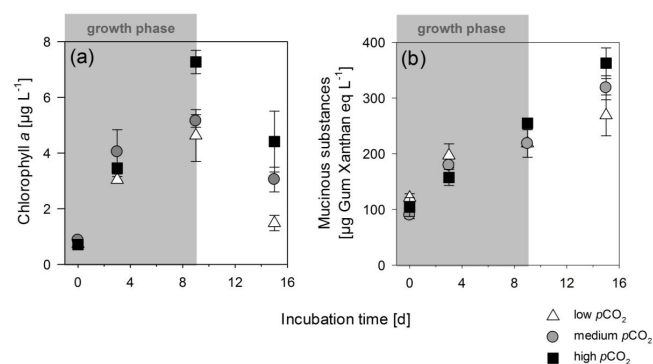
Fig. 1. Chlorophyll *a* concentration **(a)** and mucinous substance concentration **(b)** over time at low (triangle), medium (circle) and high (squares) $p\mathrm{CO_2}$. Symbols display the mean of three replicates with standard deviation.

slightly but significantly lower in low $p\mathrm{CO_2}$ treatment with $15 \pm 1.0\,\mu\mathrm{mol\,DON\,L^{-1}}$ compared to the high $p\mathrm{CO_2}$ treatment with $17 \pm 1.2\,\mu\mathrm{mol\,DON\,L^{-1}}$ ($p = 0.045$). DON decreased during the growth phase in all treatments by up to $4\,\mu\mathrm{mol\,DON\,L^{-1}}$. Differences in decrease between the single $p\mathrm{CO_2}$ treatments were not statistically significant and differences between replicates were higher than between treatments (Fig. 3). For more details on changes in DOM and $\mathrm{C:N:P}$ stoichiometry see Wannicke et al. (2012).

Total concentrations of mucinous substances ranged from 90 to $363\,\mu\mathrm{g\,Xeq\,L^{-1}}$. The total concentration doubled within the bloom phase from 90–120 μg on day 0 up to 218–$255\,\mu\mathrm{g\,Xeq\,L^{-1}}$ on day 9 (Fig. 1). From day 9 to day 15 of the experiment *Nodularia* biomass decreased, while mucinous substance concentration was still increasing. Highest increase was determined in the high $p\mathrm{CO_2}$ treatments, reaching a final concentration of $363\,\mu\mathrm{g\,Xeq\,L^{-1}}$ compared to 319 μg in the medium and $269\,\mu\mathrm{g\,Xeq\,L^{-1}}$ in the low $p\mathrm{CO_2}$ treatment. During the growth phase significantly more mucinous substances accumulated in the high $p\mathrm{CO_2}$ treatment compared to the low $p\mathrm{CO_2}$ treatment ($p = 0.039$) (Fig. 2a).

Mucinous substance concentration normalised to biomass (POC) amounted to between 3.9, 2.8 and $4.5\,\mu\mathrm{g\,Xeq\,\mu mol^{-1}}$ POC on day 0 in the low, medium and high $p\mathrm{CO_2}$ treatment and decreased over time by 49.8 %, 51.3 % and 66.8 %, respectively, during the growth phase. The high $p\mathrm{CO_2}$ treatments showed the strongest decrease in mucinous substance concentration normalised to biomass (Fig. 2c). However, this trend was not significantly correlated to $p\mathrm{CO_2}$. From day 9 to day 15, mucinous substance concentration per POC recovered to 3.3 μg in the low and the high $p\mathrm{CO_2}$ treatment and $3.5\,\mu\mathrm{g\,Xeq\,\mu mol^{-1}}$ POC in the medium $p\mathrm{CO_2}$ treatment.

For further information on gel particle size and abundance, a microscopic analysis was performed. Most cyanobacteria filaments seemed to be coated by mucus, which was stained by Alcian Blue (Fig. 4a), while CSP was mostly observed as "free" particles in the seawater (Fig. 4c, d). Additionally, free

TEP particles were observed (Fig. 4b). Due to methodological constraints it was not possible to quantify TEP or mucus separately.

The microscopic analysis revealed an increase in CSP area during the growth phase (day 0 to day 9) in all treatments (Fig. 5a). Additionally, CSP area differed significantly between low and medium ($p = 0.042$) as well as between low and high ($p = 0.009$) $p\mathrm{CO_2}$ treatments. CSP area increased by $57.0\,\mathrm{mm^2\,L^{-1}}$ ($= 32\,\%$) in the low, by $331.4\,\mathrm{mm^2\,L^{-1}}$ ($= 193\,\%$) in the medium and by $580.4\,\mathrm{mm^2\,L^{-1}}$ ($= 389\,\%$) in the high $p\mathrm{CO_2}$ treatment during the growth phase. CSP abundance clearly increased during the growth phase, i.e. by 355 %, 174 % and 148 % in the low, medium and high $p\mathrm{CO_2}$ treatment, respectively. Variability between replicates was high (standard deviation ranged between 19 % and 78 %). The average ESD of CSP differed significantly between low and high $p\mathrm{CO_2}$ treatment ($p = 0.032$). During the growth phase, CSP size decreased by $0.84 \pm 0.15\,\mu\mathrm{m}$ in the low and $0.22 \pm 0.27\,\mu\mathrm{m}$ in the medium $p\mathrm{CO_2}$ treatment, while in the high $p\mathrm{CO_2}$ treatment average ESD of CSP increased by $0.47 \pm 0.45\,\mu\mathrm{m}$ (Fig. 5c). The area covered by filaments was calculated from average filament length, width and abundance in each replicate. On day 0, filament area ranged between 131.3 and $171.6\,\mathrm{mm^2\,L^{-1}}$ and increased up to 615.3, 833.1 and $1264.4\,\mathrm{mm^2\,L^{-1}}$ in the low, medium and high $p\mathrm{CO_2}$ treatment, respectively (Fig. 5a). The increase in filament area was significantly different between low and high as well as between medium and high $p\mathrm{CO_2}$ treatments ($p = 0.014$). The increase in filament numbers was significantly different between medium vs. high and low vs. high $p\mathrm{CO_2}$ treatment ($p < 0.001$). Cyanobacteria abundance increased by 134 % in the low, 241 % in the medium and 753 % in the high $p\mathrm{CO_2}$ treatment (Fig. 5b).

3.3   Enzyme activities and recycling of organic matter

To determine the turnover of organic matter due to enzymatic cleavage outside the cyanobacterial cells, extracellular enzyme activities were measured in all samples. Enzyme activities at the start of the experiment were low and increased over time in all treatments.

The turnover of the DOP pool can be assessed through the measurement of the activity of the P-specific enzyme alkaline phosphatase (Mather et al., 2008). Alkaline phosphatase activity (APA) ranged between 62.9 and $93.6\,\mathrm{nmol\,L^{-1}\,h^{-1}}$ (V_{max}) on day 0 and increased by factor 10 until day 9 (Fig. 6). Afterwards activities remained constant or decreased slightly. Highest increase in alkaline phosphatase activity was observed in the high $p\mathrm{CO_2}$ treatment with $958.23\,\mathrm{nmol\,L^{-1}\,h^{-1}}$ on day 9.

K_{m} values of the alkaline phosphatase ranged between 0.6 and 0.9 μM at the beginning of the experiment and increased up to 2.2 μM in the low, 3.9 μM in the medium and 4.9 μM in the high $p\mathrm{CO_2}$ treatment (Table 1). As the K_{m} values
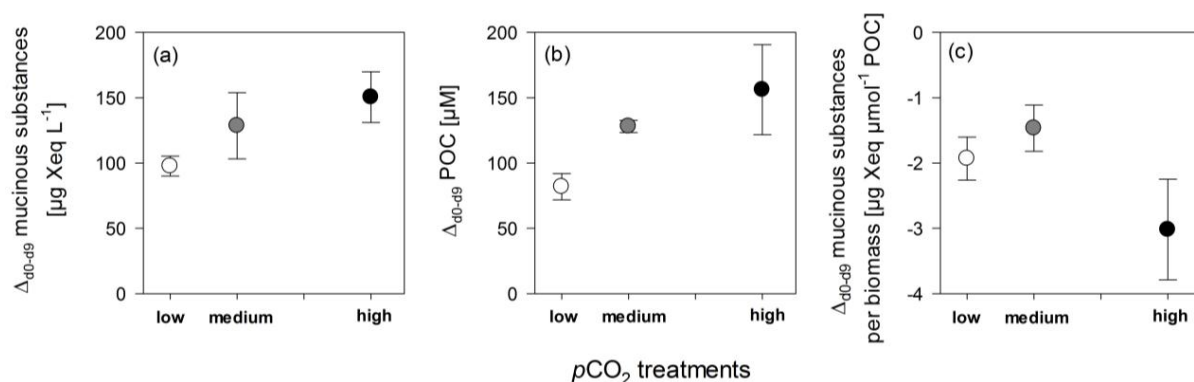
Fig. 2. Comparison of biomass production and accumulation of mucinous substances during the growth phase (day 0 to day 9). Symbols display the mean of three replicates with standard deviation.
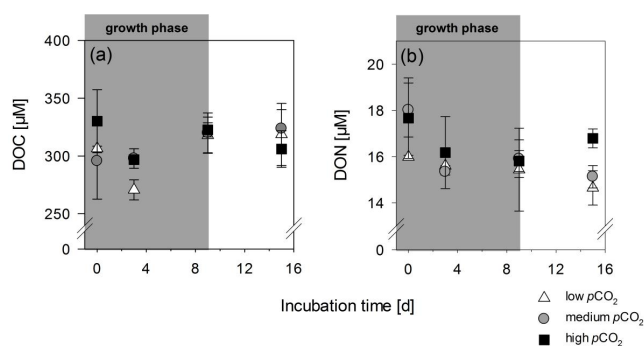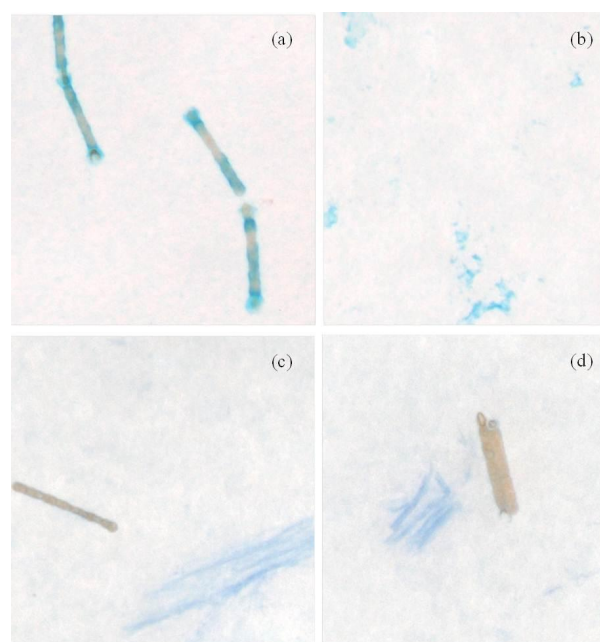

Fig. 3. DON and DOC concentrations over time at low (triangle), medium (circle) and high (squares) pCO$_2$. Symbols display the mean of three replicates with standard deviation.

Fig. 4. Microscopic view of *Nodularia spumigena* cultures covered with a mucus coating **(a)**, stained TEP **(b)**, and CSP **(c, d)**.

of the alkaline phosphatase increased with increasing pCO$_2$, the substrate affinity of the enzyme decreased.

APA showed significantly negative correlation with PO$_4^{3-}$ concentration (Table 2). On the third day of the experiment PO$_4^{3-}$ was depleted, so the cells were likely P limited. Fastest decrease and hence uptake of PO$_4^{3-}$ was observed at high pCO$_2$. From day 3 onwards, there was a net loss in DOP in all treatments (Fig. 7a). The net consumption of DOP differed between CO$_2$ treatments with 0.06 μM at low and 0.15 μM at high pCO$_2$, but differences were not significant due to high variations between replicates (Fig. 7b).

Activities of α- and β-glucosidases remained very low during the whole experiment, but increased slightly toward the end of the experiment (Table 3). On day 0, glucosidase activities remained below the detection limit. Highest activities were measured on day 15, ranging between 15.5–18.8 nmol L^{-1} h^{-1} for α-glucosidase and 16.1–18.7 nmol L^{-1} h^{-1} for β-glucosidase. Variation between replicates was higher than between treatments. Glucosidase activities are usually assigned to heterotrophic bacteria which degrade organic carbon compounds. Therefore, bacte-

rial cell-specific activities were calculated for both glucosidases (Table 3). Standard deviations were high due to high variability in bacterial cell counts between the replicates. Cell-specific activities increased over time in all treatments, with highest increase in the medium pCO$_2$ treatment. This was mainly due to a decrease in bacterial abundance over time in this treatment, while in the low and high pCO$_2$ treatments bacterial cell numbers increased slightly.

No leucine aminopeptidase (LAP) activity was detectable on day 0 ($<$ 10 nmol L^{-1} h^{-1}) but activities increased during the growth phase up to 105.17, 241.21 and 168.04 nmol L^{-1} h^{-1} in the low, medium and high pCO$_2$ treatment, respectively (Fig. 8). Highest activities

Table 1. Phosphatase activity characteristics: V_{max} is the maximum rate achieved by the system at saturating substrate concentrations in nmol per litre and hour; the Michaelis–Menten constant K_m is the substrate concentration in µM at which the reaction rate is half of V_{max}. $R^2 > 0.97$; substrate concentrations ranged from 1–150 µM; number of substrate concentrations used for each regression = 7.

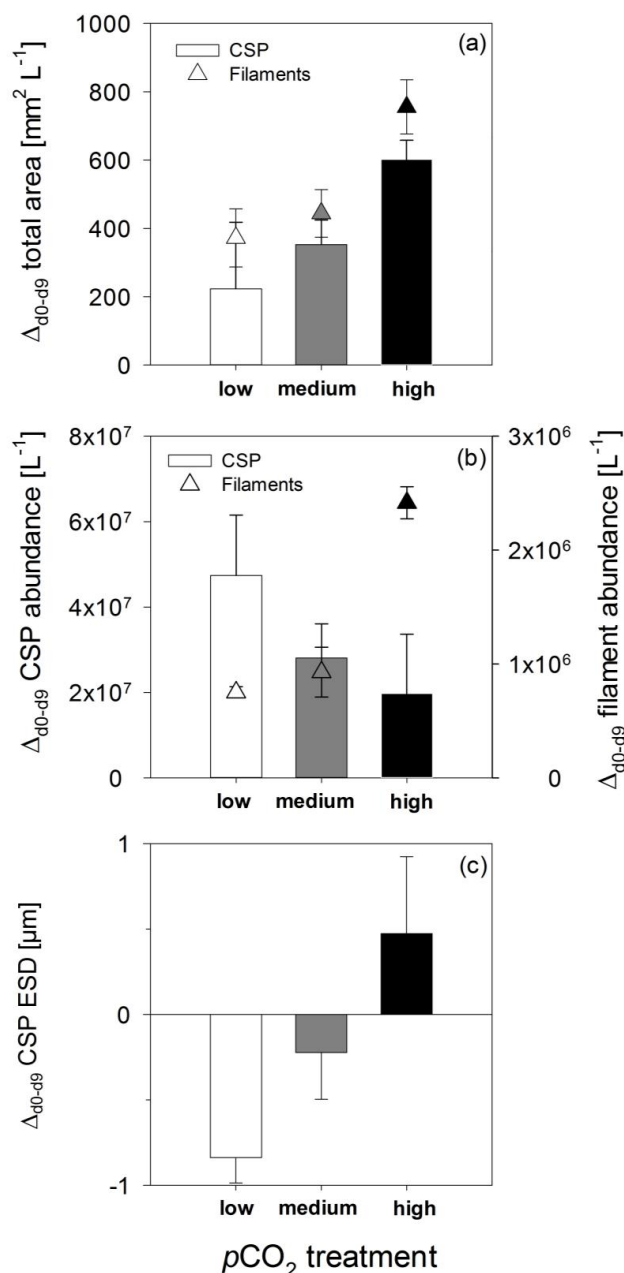| | | | pCO$_2$ treatment | | |
			low	medium	high
Day 0	V_{max}	$\frac{nmol}{L \times h}$	76.7 ± 5.8	93.6 ± 4.4	62.9 ± 2.6
	K_m	µM	0.6 ± 0.4	0.9 ± 0.3	0.7 ± 0.2
Day 9	V_{max}	$\frac{nmol}{L \times h}$	419.5 ± 17.0	693.2 ± 36.8	958.2 ± 54.5
	K_m	µM	2.2 ± 0.5	3.9 ± 1.0	4.9 ± 1.2

Table 2. Correlation of alkaline phosphatase activity (APA) with biomass of filamentous cyanobacteria (POC, Chl a), PO$_4^{3-}$, DOP, pCO$_2$, pH, N$_2$ fixation, and bacterial cell counts ($n = 36$, except for PO$_4^{3-}$ ($n = 35$) and N$_2$ fixation ($n = 32$); R = correlation coefficient). Correlation level is marked as * for low significance ($0.03 < |R| < 0.7$, $p < 0.05$), as ** for strong significance ($|R| > 0.7$, $p < 0.05$), and nc: for no correlation.

Parameter	Correlation coefficient R	Correlation
AP activity vs.		
PO$_4^{3-}$	−0.73	**
DOP	−0.64	*
POC	0.86	**
Chl a	0.73	**
pCO$_2$	−0.55	*
pH	0.55	*
N$_2$ fixation rate	−0.65	*
Bacterial cell number	0.13	nc

were measured with 268.0, 375.6 and 244.6 nmol L^{-1} h^{-1} on day 15 (Table 3). Bacterial cell-specific activities could not be calculated for leucine aminopeptidase as there is no possibility to distinguish between enzymes that are produced by cyanobacteria and heterotrophic bacteria. No significant correlation was found between APA (Table 2) or LAP (Table 4) activity and bacterial cell numbers, but both showed a significantly positive correlation with cyanobacteria biomass (POC, Chl a) and a significantly negative correlation with N$_2$ fixation rates (Table 4). Correlation between both enzymes and pCO$_2$ or pH was low.

At the end of the experiment, cyanobacteria cells were filtered out (2.7 µm GF/D filter). Extracellular enzymes in the medium, however, cannot be removed by this filtration. We determined extracellular enzyme activities of the filtrate and found that 1–2 % of the APA and 2–59 % of the LAP activity remained. Summarising our results, we can say that (1) AP was mainly attached to *Nodularia* cell surface, (2) LAP was mainly released to the medium, (3) degradation of DOP was

Fig. 5. Variation of the filaments and CSP during the growth phase (day 0 to day 9) in the low (white), medium (grey) and high (black) pCO$_2$ treatments: **(a)** total area of the filaments and CSP; **(b)** filament and CSP abundance; **(c)** equivalent spherical diameter (ESD) of CSP. Symbols and bars display the mean of three replicates with standard deviation.

driven by *Nodularia* while heterotrophic degradation of DOP was negligible, and (4) we cannot ascribe LAP activity solely to *Nodularia spumigena*. Heterotrophic bacteria might also have contributed to LAP to a certain degree although they did not build up biomass. Heterotrophic bacteria attached to the filaments may be responsible for the slight increase in
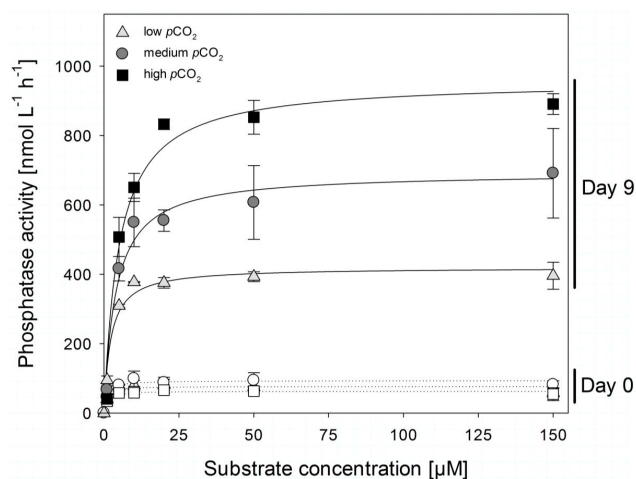
Fig. 6. Total alkaline phosphatase activity at day 0 and day 9 of low (triangle), medium (circle) and high (square) pCO$_2$ treatments. Symbols display the mean of three replicates with standard deviation.

Fig. 7. DOP concentration over time at low (triangle), medium (circle) and high (squares) pCO$_2$ **(a)** and changes from day 0 to day 9 **(b)**. Differences between treatments are not significant. Symbols display the mean of three replicates with standard deviation.
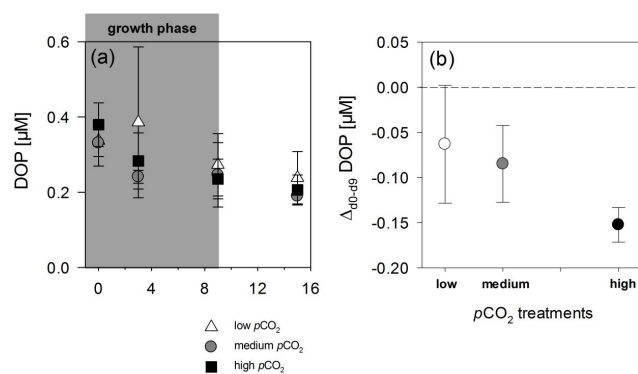
glucosidase activity towards the end of the experiment and also for some percentage of LAP activity.

4 Discussion

The aim of this study was to investigate the effect of CO$_2$-induced changes in seawater carbonate chemistry on the exudation of organic matter and its subsequent recycling by extracellular enzymes in a *Nodularia spumigena* culture. *Nodularia spumigena* growth was induced by adding phosphate on day 0 and day 3. The growth peak occurred simultaneously around day 9 in all treatments. Afterwards cell numbers declined since phosphorus was depleted. Three CO$_2$ conditions were applied by aerating the cultures with 180 ppm, 380 ppm and 780 ppm CO$_2$. Obtained pCO$_2$ values were on median 315 µatm in the low, 353 µatm in the medium, and 548 µatm in the high pCO$_2$ treatment (Wannicke et al., 2012). In general, pCO$_2$ had a stimulating effect on *Nodularia spumigena* growth and N$_2$ fixation (Wannicke et al., 2012). These findings are in accordance with earlier findings suggesting growth and POC production of cyanobacteria are increased by elevated pCO$_2$ (Hutchins et al., 2007; Barcelos e Ramos et al., 2007; Kranz et al., 2010). Findings in our study are showing increased cyanobacterial growth is in turn influencing exudation, nutrient uptake and recycling of organic matter by extracellular enzymes.

4.1 Exudation and formation of gel particles by *Nodularia spumigena*

Highest changes in mucinous substance concentration were observed at high pCO$_2$ levels (Fig. 2). Cells of *Nodularia spumigena* are covered by a mucus surface coating (Nausch,

1996; Engel et al., 2002b) that includes acidic mucopolysaccharide and is therefore stained by Alcian Blue. Thus, an increased biomass production in the high pCO$_2$ treatment leads to apparently higher mucinous substance concentrations. Field measurements revealed similar concentrations in the Baltic Sea as those found in our study (Engel et al., 2002b). In our study, when the cyanobacteria bloom declined, mucinous substance concentrations still increased (Fig. 1); this was pronounced at high pCO$_2$. In accordance with the accumulation of mucinous substances, the increase in POC and Chl *a* concentration was significantly higher with increasing pCO$_2$ during the growth phase (Fig. 2). Yet, the ratio of mucinous substances to POC concentration was decreasing during the growth phase and increasing afterwards. This indicates that biomass production was more stimulated than carbon exudation and mucus production during the first nine days of the incubation. As growth slowed down and filament numbers decreased, more carbon was released either by active exudation or cell lysis. Therefore, our conclusion is that cell-specific production of mucinous substances is not effected by CO$_2$ but due to higher biomass, as we found more mucinous substances in the high pCO$_2$ treatments.

Due to methodological constraints, we cannot discriminate between acidic polysaccharides contained in TEP and in mucus. While TEP is formed by coagulation and aggregation of exuded DOC, large mucopolysaccharide molecules are released by cells and coagulate in the mucus layer (Nausch, 1996). It has been proposed that the mucus layer has the function to protect the autotrophic cells against degradation by heterotrophic bacteria and help the cells to aggregate (O'Neil and Roman, 1992). Thus, it would be interesting to investigate in further studies which factors influence the production of mucus and to quantify the amount per cell. For heterotrophic bacteria it is known that maximal extracellular polymeric substance production occurs under nutrient limitation in the presence of an excess carbon source

Table 3. Glucosidases and leucine aminopeptidase activities at different pCO$_2$ (V_{max} and standard deviation; $n = 3$) on day 15 (= day of highest activities).
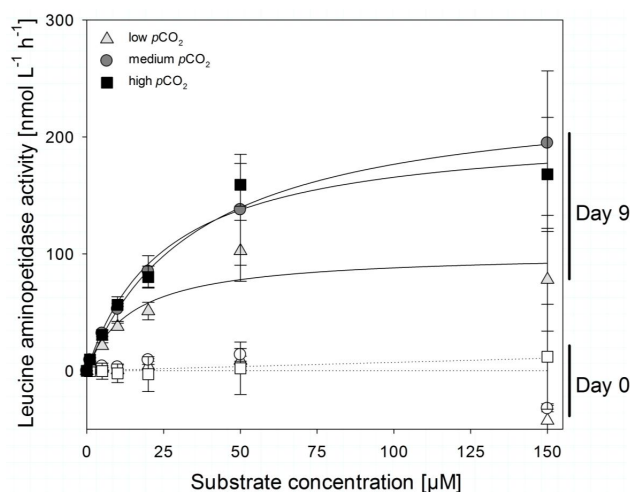
Enzyme			pCO$_2$ treatment		
			low	medium	high
α-glucosidase	total	$\frac{\mathrm{nmol}}{\mathrm{L} \times \mathrm{h}}$	15.6 ± 2.8	15.5 ± 2.7	18.8 ± 7.9
	per cell	$\frac{\mathrm{amol}}{\mathrm{L} \times \mathrm{h}}$	28.5 ± 19.9	77.6 ± 22.6	46.9 ± 51.2
β-glucosidase	total	$\frac{\mathrm{nmol}}{\mathrm{L} \times \mathrm{h}}$	18.7 ± 2.4	16.1 ± 2.9	18.1 ± 7.1
	per cell	$\frac{\mathrm{amol}}{\mathrm{L} \times \mathrm{h}}$	33.0 ± 21.1	81.4 ± 14.7	42.9 ± 44.7
leucine aminopeptidase	total	$\frac{\mathrm{nmol}}{\mathrm{L} \times \mathrm{h}}$	268.0 ± 133.8	375.6 ± 60.7	244.6 ± 58.2

Table 4. Correlation of leucine aminopeptidase activity with biomass of filamentous cyanobacteria (POC, Chl a), dissolved inorganic and organic P, pCO$_2$, pH, N$_2$ fixation and bacterial cell counts ($n = 36$, except for NO$_3$, DON ($n = 35$) and N$_2$ fixation ($n = 32$); R = correlation coefficient). Correlation level is marked as * for low significance ($0.03 < |R| < 0.7$, $p < 0.05$) as ** for strong significance ($|R| > 0.7$, $p < 0.05$), and nc: for no correlation.

Parameter	Correlation coefficient R	Correlation
LAP activity vs.		
NO$_3$	-0.46	*
DON	-0.34	*
POC	0.71	**
Chl a	0.52	*
pCO$_2$	-0.63	*
pH	0.64	*
N$_2$ fixation rate	-0.73	**
Bacterial cell counts	0.03	nc

Fig. 8. Aminopeptidase activities on day 0 and day 9 of low (triangle), medium (circle) and high (square) pCO$_2$ treatments. Symbols display the mean of three replicates with standard deviation.

(Sutherland, 1985). In *Nostoc*, a filamentous N$_2$-fixing freshwater cyanobacterium, extracellular polymeric substances serve as a sink for the excess fixed carbon under unbalanced C:N metabolism (Otero and Vincenzini, 2004). This mucus production happens especially under high pCO$_2$ conditions, where the potential rate of CO$_2$ fixation exceeds that of N$_2$ fixation. In our study, ratios of fixed CO$_2$ to fixed N$_2$ were higher than the Redfield ratio with maximum values in the medium pCO$_2$ treatment (Wannicke et al., 2012), which does not suggest potential higher production of mucus in the high pCO$_2$ treatments. Hence, we suggest that principally more TEP, and not mucus, is formed at higher pCO$_2$ after growth decelerated.

In contrast to TEP, CSP are barely stained in the mucus surface coating of *Nodularia*, so CSP can be considered as discrete, protein-containing particles during this experiment. CSP area was significantly positive correlated with pCO$_2$. CSP abundance increased over time in all treatments with highest increase in the low pCO$_2$ treatment. The average size of the individual CSP decreased at low and medium pCO$_2$

but increased at high pCO$_2$ (Fig. 5). This indicates that CSP were possibly degraded in the low and the medium pCO$_2$ treatment to more but smaller particles while more material was aggregating to bigger particles in the high pCO$_2$ treatment. This might be explained by increasing stickiness and changing composition of the organic matter and especially by a higher concentration of acidic sugars (Alldredge et al., 1993; Engel et al., 2004a), which could also hint towards the formation of free TEP.

Transferred to the field, an increase in gel particles would change the aggregation of filaments and the export of organic matter towards deeper layers of the ocean. Additionally, it may also effect bacterial growth as mucus is a suitable substrate for marine bacteria. However, the composition changes of organic matter, especially sugars and amino acids, under elevated pCO$_2$ need to be investigated more in detail to proof this assumption.

4.2 Production and subsequent recycling of dissolved organic matter by extracellular enzymes

Some studies propose that N$_2$ fixation and subsequent release of DON may be a mechanism to cope with excess light energy on a short timescale (Lomas et al., 2000; Wannicke et al., 2009). So far, no report on the effect of pCO$_2$ on DON release exists, while exudation of DOC was shown to be stimulated with increasing pCO$_2$ (Borchard and Engel, 2012). In the present study, DOC concentrations slightly increased in all treatments during the growth phase, while DON and DOP concentrations decreased (Figs. 3 and 7). Our results indicate that the algae were releasing DOC to the seawater but we cannot confirm a stimulating effect of elevated pCO$_2$ on DOC exudation. In contrast, DON concentrations decreased, which may be explained by enzymatic degradation to acquire nitrogen. As CSP area was increasing during our experiment and with pCO$_2$, the DON loss may also be explained by aggregation processes to CSP. The growth of *Nodularia spumigena* after phosphate depletion on day 3, as well as the continuing POP formation between days 3 to 15 (Unger et al., 2012), indicated the utilization of DOP as organic source for phosphorus. Further aspects of changes in DOM and stoichiometry of particulate organic matter are discussed in Wannicke et al. (2012). Unger et al. (2012) describe in detail changes in the turnover of P and the composition of the P pool in our study.

Different compounds of organic matter may be released by primary producers under elevated pCO$_2$ but also extracellular enzymes may modulate the composition and properties of organic matter. To determine the turnover rates of organic matter due to enzymatic cleavage, extracellular enzyme activities of four key enzymes in carbon and nutrient cycling (alkaline phosphatase, α- and β-glucosidase, and leucine aminopeptidase) were followed over time in all treatments. Enzyme activities at the start of the experiment were low and increased over time in all treatments.

Leucine aminopeptidase is a major enzyme in microbial degradation of organic matter. Most of its activity was found to be associated with particles (Hoppe et al., 2002). *Nodularia spumigena* is able to express leucine aminopeptidase to degrade proteins (Stoecker et al., 2005) and to acquire amino acids. In terms of magnitude, LAP activities determined during this study are in the range of those previously measured in the Baltic Sea (Nausch, 1998b). Nausch and co-workers determined an average activity of 263.1 ± 128.4 nmol L^{-1} h^{-1} in autumn in the Pomeranian Bight. In our study, leucine aminopeptidase activity (LAP) positively correlated with cyanobacterial biomass and negatively with N$_2$ fixation rates (Table 4). N$_2$ fixation decreased over time in all treatments while LAP activity increased. In the end of the vegetation period, senescent algae exude polymeric substances, such as proteins, which have to be enzymatically cleaved before uptake (Hoppe et al., 2002). This might explain the temporal increase in leucine aminopeptidase activity in our study

especially when *Nodularia* stopped growing and filament numbers decreased. We conclude that *Nodularia* might have changed their N acquisition strategy from N$_2$ fixation towards enzymatic degradation of DOM over time.

Enzyme activities may respond quickly to changes in pH but also in substrate availability and their kinetics are modified to benefit best from environmental conditions. In this context, it is important to distinguish between direct pCO$_2$- and pH-effects on microbial activity from effects induced by CO$_2$-related changes in autotrophic organic matter production. Piontek et al. (2010) suggest that rates of enzymatic hydrolysis by marine glucosidase assemblage are not at their maximum at present-day seawater pH. Our results show an indirect effect of increasing pCO$_2$ on LAP activities due to increased cyanobacterial growth. Correlation between LAP activity and pCO$_2$ was low. However, a direct pH effect could, in theory, have been masked by differences in the quantity or nature of enzymes and their substrates, but such effects cannot be detected with the experimental methods used here. This is in good accordance with previous results from a mesocosm study where increasing pCO$_2$ changed the photosynthetic production of dissolved and particulate organic matter and therefore increased enzyme activities (Grossart et al., 2006).

In our study, initial PO$_4^{3-}$ concentrations were below 0.5 µM and APA was negatively correlated to PO$_4^{3-}$ concentration over time (Table 2). It is well documented that under P-limiting conditions, *Nodularia spumigena* has a high uptake affinity for DOP by production and release of alkaline phosphatase (Nausch, 1998a; Wu et al., 2012). A direct relationship between intracellular PO$_4^{3-}$ concentration and APA was shown in Huber and Hamel (1985a) and indicated that cellular rather than external phosphorus sources controlled APA. A threshold of APA around 1 µM PO$_4^{3-}$ was defined (Nausch, 1998a). In terms of magnitude, APA determined during this study is comparable with data given by Nausch (1998a) for the Baltic Sea (5–550 nM h^{-1}). We conclude that *Nodularia* expressed more alkaline phosphatase to acquire phosphorus from organic sources when PO$_4^{3-}$ concentrations declined.

We found increased APA by $\sim$ factor 1.64 and 2.25 in the medium and high compared to the low pCO$_2$ treatment (Fig. 6). Correlation between pH and the increase of APA was low (Table 2). The alkaline phosphatase has an optimum between pH 7.5 and 10 (e.g. Healey and Hendzel, 1979; Münster et al., 1992), depending on origin and composition of the enzyme assemblage. Other enzymes were shown to have an optimum at a pH below present seawater pH (e.g. Münster et al., 1992; Grossart et al., 2006; Piontek et al., 2010). Therefore, we assume that pH may also have a direct stimulating effect on APA.

Increased APA in the high pCO$_2$ treatments may have supported the growth of *Nodularia* by reducing P limitation. While APA was increasing, DOP concentrations decreased in

parallel (Fig. 7). The rapid hydrolysis and recycling of DOP by *Nodularia* may have stimulated POP (biomass) production. On the other hand, increased C availability in the high pCO$_2$ treatments may have stimulated *Nodularia* growth, which in turn increased the expression of more alkaline phosphatase and this consecutively supported *Nodularia* growth. This point needs further examination as it cannot be resolved completely here. Our results are in accordance with another study that investigated the effect of elevated pCO$_2$ on APA during a mesocosm experiment under phosphate depletion (Tanaka et al., 2008). To sum up, we hypothesize that DOP recycling by alkaline phosphatase was stimulated by increasing pCO$_2$ and likely beneficial for *Nodularia* growth.

The K_m values of APA in our experiment increased over time and tended to be higher at high pCO$_2$ but differences were not significant (Table 1). Higher K_m values indicate decreasing substrate affinity. Enzymes catalyzing the same reaction may occur in more than one molecular form (isoenzymes), characterised by different half-saturation constants (K_m), temperature, and pH optima (Hoppe, 2003). Substrate specificity of phosphatase is scarcely explored. The increasing K_m values in our study might be explained by the production of different and less-specific isoenzymes of the alkaline phosphatase. This could be a mechanism for *Nodularia* to access more and different compounds of the DOP pool under severe P-limitation. Because total APA and K_m values were likewise increasing, enzyme efficiency ($V_{max} : K_m$ ratio) did not increase with increasing pCO$_2$. In terms of magnitude K_m values determined here are in the range of values measured in the Central North Pacific Ocean (Perry and Eppley, 1981) and in the Gulf of Biscay (Labry et al., 2005) but below values from the Baltic Sea, which ranged between 4 and 37 µM (Grönlund et al., 1996). In conclusion, phosphate affinity of the alkaline phosphatase tended to be lower in the high pCO$_2$ treatment but enzyme efficiency remained.

To investigate the effect of pCO$_2$ on production and exudation of organic matter by *Nodularia*, we tried to exclude heterotrophic bacteria to avoid fast degradation of labile components. Although a low number of heterotrophic bacteria were still present in the treatments, we did not detect active growth. In our study, AP and LAP activities could be mostly assigned to cyanobacteria although we cannot completely exclude the contribution of heterotrophic bacteria to LAP activity. This is in accordance with previous studies that found extracellular enzyme activity in association with *Nodularia* sp. (Huber and Hamel, 1985b; Stoecker et al., 2005). Around 37 % of APA and 30 % of LAP activity were associated with cyanobacteria during a *Nodularia* dominated bloom in the Baltic Sea (Stoecker et al., 2005). However, heterotrophic bacteria may have contributed to organic matter degradation, which is reflected in slightly increasing α- and β-glucosidase activities over time. In the field, heterotrophic bacteria may compete with cyanobacteria for nutrients and contribute significantly to the recycling of organic matter, especially in the decline of algal blooms (Chrost et al., 1989;

Jacquet et al., 2002). However, cyanobacteria are efficient in using DOP and therefore successful competitors with heterotrophic bacteria for phosphate (Vahtera et al., 2007; Michelou et al., 2011).

4.3 Implications for the Baltic Sea under environmental change

Ocean acidification is only one of several environmental changes caused by rising atmospheric CO$_2$ concentrations. To provide a realistic picture of climate change for the marine ecosystem, the multiple effects of environmental change need to be addressed (Rost et al., 2008). Combined effects of climate change (e.g. CO$_2$ and temperature) may compensate or amplify direct effects of increasing CO$_2$ levels alone. Similar to pH, temperature may affect stability, hydrolyzation rates and substrate affinity of enzymes (Hernandez et al., 2002). Temperature may also enhance diazotrophic growth as suggested by O'Neil et al. (2012) and consequently expand range and timing of diazotrophic cyanobacteria blooms in the future. Higher temperatures will also reduce the mixing of the upper ocean due to increased thermal stratification and suppress the upwelling of nitrate (Doney, 2006), further promoting the growth of diazotrophic organisms such as *Nodularia spumigena*.

On the other hand, the on-going increase in global N loading may potentially increase P limitation in marine ecosystems (Dyhrman et al., 2007). P is an important driver of microbial dynamics in marine systems which lead to an intense competition for P between phototrophic and heterotrophic microorganism and forces them to improve their P-cycling efficiencies. As *Nodularia* is able to utilize DOP more efficiently compared to other phytoplankton in the Baltic Sea (Vahtera et al., 2010), they may benefit from decreasing seawater pH and possibly also from ocean warming. This may promote a succession of cyanobacterial communities towards this genus. It was recently shown that the cyanobacterium *Aphanizomenon ovalisporum* promotes inorganic phosphate supply by secreting toxins, which induces alkaline phosphatase in other phytoplankton species (Bar-Yosef et al., 2010; Raven, 2010). Some *Nodularia* strains produce the cyanotoxin nodularin during the summer bloom (Sivonen et al., 1989; Sellner et al., 2003), which accumulates in blue mussels (Kankaanpää et al., 2007), flounders (Kankaanpää et al., 2005) and seabirds (Sipiä et al., 2004). Mass occurrences of *Nodularia* due to climate change might lead to an overall increase in cyanotoxin concentration. These toxins might spread further in the ocean and accumulate in the invertebrates and vertebrates with unknown consequences for the marine food web and eventually humans. Furthermore, extended blooms may lead to higher aggregation and export of filaments to deeper waters. Enhanced microbial degradation there could enhance oxygen consumption and consequently expand the already existing oxygen-deficient zone in the deeper waters of the Baltic Sea.

5   Conclusions

Our results suggest that *Nodularia spumigena* can grow faster under elevated pCO$_2$ supported by enhanced recycling of organic matter to satisfy nutrient demands. Aggregation of filaments and the export of organic matter towards deeper water layers may change due to the formation of more gel particles by *Nodularia spumigena*. To predict the effect of climate change on cyanobacterial dynamics and organic matter cycling in the sea, we need to broaden the focus by including the combined effects of all projected changes in environmental conditions.

Supplementary material related to this article is available online at: http://www.biogeosciences.net/10/ 567/2013/bg-10-567-2013-supplement.zip.

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