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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* has a high enzyme affinity for dissolved organic phosphorus (DOP) by production and release of alkaline phosphatase. Additionally, it is 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 aerated with three different pCO_2 levels corresponding to values from glacial periods to future values projected for the year 2100. 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 was influencing exudation and 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 and simultaneously increasing exudation. During the growth phase significantly more mucinous substances accumulated in the high pCO_2 treatment reaching 363 µg Gum Xanthan eq l^{-1} compared to 269 µg Gum Xanthan eq 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. In conclusion, our results suggest that *Nodularia spumigena* can grow faster under elevated pCO_2 by enhancing the recycling of organic matter to acquire nutrients.

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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 and diazotrophic *Nodularia spumigena* (Sellner, 1997). Some *Nodularia* strains produce the cyanotoxin nodularin during the summer bloom, which is harmful to humans (Sivonen et al., 1989; Sellner et al., 2003). As nodularin accumulates in blue mussels (Kankaanpää et al., 2007), flounders (Kankaanpää et al., 2005) and seabirds (Sipiä et al., 2004), *Nodularia spumigena* has an ecological impact on the Baltic Sea food web. Another important aspect is that *Nodularia* have 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 (~ 200 Gg N yr^{-1}) (Larsson et al., 2001; Schneider et al., 2003). Thus, *Nodularia* is of high biogeochemical importance for this region.

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 utilization of dissolved organic phosphorus (DOP) (Nausch, 1998; Nausch and Nausch, 2006; Björkman and Karl, 1994). Because the ability to fix N_2 is controlled by phosphate and trace metal bioavailability (Paytan and McLaughlin, 2007), the availability of DOP may be an important factor influencing the distribution of diazotrophic cyanobacteria in the Baltic Sea (Niemi, 1979). 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 (Nausch et al., 2004; Kononen et al., 1992). 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

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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).

Transparent exopolymer particles (TEP) 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). 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 TEP 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* (Wannicke et al., 2009). Up to 89 % of the fixed nitrogen and 53 % of the fixed carbon were released during the light period. Nevertheless, it is not clear which factors regulate the exudation of DON in cyanobacteria (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 (Otero and Vincenzini, 2004; Nausch, 1996).

Microbes consume carbohydrates and proteins to meet their energy and nutrient requirements and to build up biomass. Therefore, they hydrolyse high molecular weight 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

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alkaline phosphatase catalyses the hydrolytic break-down of the PO_4^{3-} end from dissolved organic matter and is expressed in response to phosphate limitation in many bacterial and phytoplankton species (Azam et al., 1983; Chrost and Overbeck, 1987; Labry et al., 2005; Beardall et al., 2001). 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 pCO_2 the ocean pH decreases 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 increased since glacial periods from 180 ppm to 380 ppm nowadays and, for the future, we expect values around 780 ppm for 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 little 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 been 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 changes and therefore the microbial driven biogeochemical processes will probably not change dramatically (Joint et al., 2010). On the other side, it has been shown that pH has a regulating effect on enzyme activities. 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 pCO_2 (Grossart et al., 2006).

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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 normalized to cell abundance (Engel et al., 2004a). Increasing pCO_2 may potentially effect N_2 fixation and increase the release of dissolved organic matter 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 is only one study published on the effects of increasing CO_2 on *Nodularia* spp. (Czerny et al., 2009). Yet observations on changes in extracellular enzyme activities and turnover of organic matter in cyanobacteria blooms due to ocean acidification are still lacking.

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

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

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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 enough 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 316 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 549 µatm (Wannicke et al., 2012).

The design of the batch culture experiment is described in more detail in Wannicke et al. (2012). Briefly, 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 were added at the beginning of the experiment (day 0) and another 0.5 µM PO_4 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}.

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2.2 Carbonate chemistry

The carbonate chemistry was determined by measuring pH and total dissolved inorganic carbon (TC) 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. TC 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 $\pm 2\,\mu mol\,kg^{-1}$. pCO_2 and total alkalinity (TA) were calculated with CO2SYS (Lewis et al., 1998).

2.3 Analysis of inorganic and organic compounds

Determination of nutrients (NH_4^+, NO_3^- and PO_4^{3-}) was accomplished by filtering 60 ml sample through a combusted GF/F filter and measured colorimetrically in a spectrophotometer U 2000 (Hitachi-Europe GmbH, Krefeld, Germany) according to the method of Grasshoff et al. (1983). Ammonium concentrations remained undetectable in 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

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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 oxidized with an alkaline peroxodisulfate solution (Grasshoff et al., 1983) in a microwave (μPrep-A) to convert organic phosphorus into DIP. The DIP 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 dissolved inorganic phosphorous (DIP) 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 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 of 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

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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 is staining 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 triplicates. 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 spectrophotometric 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_2SO_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 (eq), as xanthan gum was used for calibration.

For microscopic analysis of mucinous substances size and abundance, 5 ml samples were filtered in duplicates onto 0.4 µm Nuclepore filters, stained and incubated with 1 ml of Alcian Blue for 3 s, and rinsed with distilled water. Samples for CSP size and abundance were processed identically except that they were stained and incubated with 1 ml of Coomassie Blue solution for 30 s. CSP are barely stained in the mucus surface coating of *Nodularia* so CSP can 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 analyzed 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

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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 gum xanthan equivalents were 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 bacterial 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 is no significant substrate hydrolysis due to abiotic processes control samples for every concentration of substrate added were measured with distilled water or sterilized 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 bacterial extracellular enzymes was calculated as the maximum hydrolysis rate (V_{max}) using the software SigmaPlot 12.0. The specific alkaline phosphatase activity (sAPA) gives the hydrolysis rate of the substrate per µg chlorophyll-*a* and hour. The Michaelis–Menten constant K_m is the substrate concentration at which the reaction rate is half of V_{max}.

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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 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. 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 pair(s) 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 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 and cell productivity is given in Wannicke et al. (2012) and will only briefly be summarized here. *Nodularia* 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 in the low and 7.3 µg l^{-1} in the high pCO_2 treatment on day 9 (Fig. 1). We refer to this period as the "growth phase". From day 9 to day 15, chl *a* 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

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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. 3). 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. No significant growth of heterotrophic bacteria was observed over time and cell numbers remained around 5×10^5 cells l^{-1}.

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. 2). The average DON concentration was slightly but significantly lower in low pCO_2 treatment with 15 ± 1.0 µM compared to the high pCO_2 treatment with 17 ± 1.2 µmol DON l^{-1} (p = 0.045). DON decreased during the growth phase in all treatments by up to 4 µmol DON l^{-1}. Differences in decrease between the single pCO_2 treatments were not statistically significant and differences between replicates were higher than between treatments (Fig. 2). For more details on changes in DOM and $C : N : P$ stoichiometry see Wannicke et al. (2012).

Total concentrations of mucinous substances ranged from 90 to 363 µg Gum Xanthan eq l^{-1}. The total concentration doubled within the bloom phase from 90–120 µg on day 0 up to 218–255 µg Gum Xanthan eq 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 pCO_2 treatments reaching a final concentration of 363 µg Gum Xanthan eq l^{-1} compared to 319 µg in the medium and 269 µg Gum Xanthan eq l^{-1} in the low pCO_2 treatment. During the

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growth phase significantly more mucinous substances accumulated in the high pCO_2 treatment compared to the low pCO_2 treatment ($p = 0.039$) (Fig. 3a).

Mucinous substance concentration normalized to biomass (POC) amounted between 3.9, 2.8 and 4.5 µg Gum Xanthan eq µmol^{-1} POC on day 0 in the low, medium and high pCO_2 treatment and decreased over time by 49.8 %, 51.3 % and 66.8 %, respectively, during the growth phase. The high pCO_2 treatments showed the strongest decrease in mucinous substance concentration normalized to biomass (Fig. 3c). However, this trend was not significantly correlated to pCO_2. From day 9 to day 15 mucinous substance concentration per POC recovered to 3.3 µg in the low and the high pCO_2 treatment and 3.5 µg Gum Xanthan eq µmol^{-1} POC in the medium pCO_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.

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 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 pCO_2 treatment with 958.23 nmol l^{-1} h^{-1} on day 9.

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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 pCO_2 treatment (Table 1). As the K_m values of the alkaline phosphatase increased with increasing pCO_2, the substrate affinity of the enzyme decreased.

The chl *a* specific alkaline phosphatase activity (sAPA) decreased from day 0 to day 3 and then increased as inorganic P became depleted. During the growth phase sAPA decreased in average in the low pCO_2 treatment by -11.2 nmol µg chl a^{-1} h^{-1}, while values in the medium and high pCO_2 treatments increased by 27.7 and 43.5 nmol µg chl a^{-1} h^{-1}.

No correlation was found between APA and heterotrophic bacterial cell numbers (Table 2). Furthermore, APA declined strongly after filtering the cyanobacteria cells out at the end of the experiment, which indicates, that the enzyme was mainly associated to the presence of *Nodularia spumigena*. APA showed significantly positive correlation with biomass (POC and chl *a*) and significantly negative correlation with PO_4 concentration (Table 2). No strong correlation between APA and pCO_2 could be observed.

On the third day of the experiment inorganic phosphorus was depleted, so the cells were likely P limited. Fastest decrease and hence uptake of DIP was observed at high pCO_2. From day 3 onwards, there was a net loss in DOP (Fig. 7). The net consumption of DOP differed significantly between CO_2 treatments with 0.06 µM at low and 0.15 µM at high pCO_2 ($p = 0.038$).

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. Glucosidase activities are usually assigned to heterotrophic bacteria which degrade organic carbon compounds. Therefore, bacterial 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.

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No leucine aminopeptidase (LAP) activity was detectable on day 0 ($< 10\,\mathrm{nmol\,l^{-1}\,h^{-1}}$) but activities increased during the growth phase up to 105.17, 241.21 and $168.04\,\mathrm{nmol\,l^{-1}\,h^{-1}}$ in the low, medium and high pCO_2 treatment (Fig. 8). Highest activities were measured with 268.0, 375.6 and $244.6\,\mathrm{nmol\,l^{-1}\,h^{-1}}$ 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 or heterotrophic bacteria. As growth of heterotrophic bacteria and productivity was low, we assumed that LAP activity was principally due to cyanobacteria. No correlation was found between LAP activity and bacterial cell numbers (Table 4). However, LAP showed a significantly positive correlation with cyanobacteria biomass (POC) and a significantly negative correlation with N_2 fixation rates (Table 4). No direct correlation between LAP activity and pCO_2 or pH was observed.

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 average 316 µatm in the low, 353 µatm in the medium, and 549 µatm in the high pCO_2 treatment (Wannicke et al., 2012). CO_2 treatment–related effects were identified for cyanobacterial growth which in turn was influencing exudation, nutrient uptake and recycling of organic matter by extracellular enzymes.

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 that suggested growth and POC production of cyanobacteria to be increased by elevated pCO_2. (Hutchins et al., 2007; Kranz et al., 2010; Barcelos e Ramos et al.,

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2007). 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 term scale (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 et al., 2011). In the present study, DOC concentrations slightly increased in all treatments during the growth phase, while DON concentrations decreased. Our results indicate that the algae were releasing organic carbon compounds to the seawater but we cannot confirm a stimulating effect of elevated pCO_2 on exudation. In contrast, dissolved organic nitrogen concentrations decreased which may be explained by enzymatic degradation to acquire nitrogen or aggregation as CSP. Further aspects of changes in DOM and stoichiometry of particulate organic matter are discussed in Wannicke et al. (2012).

Highest changes in mucinous substance concentration were observed at high pCO_2 levels. Cells of *Nodularia spumigena* are covered by a mucus surface coating (Engel et al., 2002b; Nausch, 1996) 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 found in our study (Engel et al., 2002b). When the cyanobacteria bloom declined, mucinous substance concentrations still increased.

In accordance with the accumulation of mucinous substances, the increase in POC and chl *a* concentrations was significantly higher with increasing pCO_2 during the growth phase. 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. Due to methodological constraints, we cannot discriminate between acidic polysaccharides contained in TEP and in mucus. While TEP is formed by coagulation and aggregation

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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 (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 present pCO_2 treatment (Wannicke et al., 2012) which is not explaining 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 but increased at high pCO_2. 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 particles would change the export of organic matter towards deeper layers of the ocean. However, the composition changes of organic matter, especially sugars

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and amino acids, under elevated pCO_2 need to be investigated more in detail to proof this assumption.

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 the four major enzymes, 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. The growth of *Nodularia spumigena* after phosphate depletion on day 3, as well as the continuing POP formation between days 3 to 15 (data not shown), indicated the utilization of DOP. 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, 1998; Wu et al., 2012). In our study, both, alkaline phosphatase and leucine aminopeptidase activities could be assigned to cyanobacteria as heterotrophic bacterial cell numbers were low and no cell growth was observed. 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). A direct relationship between intracellular PO_4 concentration and APA was shown (Huber and Hamel, 1985a) indicating that cellular rather than external phosphorus controlled APA and a threshold around 1 µM was defined (Nausch, 1998). In our study, initial PO_4 concentrations were below 0.5 µM and APA was negatively correlated to PO_4 concentration which fits well into the mentioned theory. In terms of magnitude, our measured APA is comparable with those given by Nausch (1998) who stated an annual average APA of 5–550 $nM\,h^{-1}$ for the Baltic Sea.

The chl *a* specific alkaline phosphatase activity (sAPA) is a tool to determine phosphorus availability and limitation of phytoplankton and bacteria as suggested by Tanaka et al. (2006). The sAPA decreased from day 0 to day 3 after addition of inorganic P and

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then increased since inorganic P was depleted. During the growth phase sAPA decreased in the low pCO_2 treatment, while values in the medium and even more in the high pCO_2 treatments increased. In terms of magnitude our sAPA fits into the range determined by Vahtera et al. (2007) for *Nodularia spumigena* cultures with 46.69–269.48 nmol µg chl a^{-1} h^{-1} under P-limitation. Stoecker and co-workers (2005) determined similar rates in axenic cultures but higher rates around 6.5–14 µmol µg chl a^{-1} h^{-1} during a cyanobacteria bloom in the Baltic Sea.

The K_m values of APA in our experiment increased over time and with increasing pCO_2. Higher K_m values indicate decreasing substrate affinity. Enzymes catalyzing the same reaction may occur in more than one molecular form (isoenzymes), characterized by different half-saturation constants (K_m), temperature and pH optima (Hoppe, 2003). Substrate specificity of phosphatase is little 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. However, enzyme efficiency ($V_{max} : K_m$ ratio) did not increase with increasing pCO_2 because total APA and K_m values were likewise increasing. 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).

No CO_2 effects were determined regarding the total uptake of nitrate and phosphate. For phosphate, this result was not surprising because phosphorus was the least available element and likely to become exhausted quickly in all treatments. The increase of APA in the medium and high compared to the low pCO_2 treatment (~ factor 1.64 and 2.25, respectively) could not be directly correlated to the pH dependency of enzyme activities. The main explanation probably lies in the increased growth of *Nodularia* and consequently their response to phosphate depletion. This is supported by the parallel drop in DOP, indicating that this pool was rapidly hydrolysed and recycled by *Nodularia* which then stimulated POP production. Unger et al. (2012) describe in detail changes

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in the turnover of P and the composition of the P pool. Our results are in accordance with another study investigating the effect of elevated pCO_2 on APA during a mesocosm experiment under phosphate depletion (Tanaka et al., 2008). Specific phosphate affinity and specific APA tended to be higher in the high pCO_2 treatment although no statistical differences were found.

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 our study, leucine aminopeptidase activity (LAP) positively correlated with cyanobacterial biomass (chl *a* and POC) and negatively with N_2 fixation rates. N_2 fixation decreased over time in all treatments while LAP activity increased. This proposes that *Nodularia* might have changed their N acquisition strategy from N_2 fixation towards enzymatic degradation of DOM over time. No correlation could be determined between LAP activity and pCO_2. This is in contrast to the results of a mesocosm study were protease activity was significantly higher in the high pCO_2 mesocosm compared with protease activity in the medium and low mesocosms (Grossart et al., 2006). However, a 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. In terms of magnitude our activities are in the range of those previously measured in the Baltic Sea (Nausch et al., 1998). Nausch and co-workers determined an average activity of $263.1 \pm 128.4\,\mathrm{nmol\,l^{-1}\,h^{-1}}$ in autumn in the Pomeranian bight. 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 also explain the temporal increase in leucine aminopeptidase activity in our study especially when *Nodularia* stopped growing and filament numbers decreased. The activity of α- and β- glucosidases activities was low or even not detectable, presumably because there were enough monosaccharides available and/or the bacterial community was not active.

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Enzyme activities may respond quickly to changes 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 and coworkers (2010) suggest that rates of enzymatic hydrolysis by marine glucosidase assemblages are not at their maximum at present-day seawater pH. Our results show an indirect effect of increasing pCO_2 on enzyme activities due to increased cyanobacterial growth. This is in good accordance with previous results from a mesocosm study where pCO_2-induced changes in photosynthetic production of dissolved and particulate organic matter and therefore increased enzyme activities were found (Grossart et al., 2006).

To investigate the effect of pCO_2 on production and exudation of organic matter, 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 conclusion, degradation of organic matter by heterotrophic bacteria is negligible, which is supported by low to not detectable α- and β-glucosidase activities. 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 and other inorganic nutrients (Michelou et al., 2011; Vahtera et al., 2007).

Ocean acidification is only one aspect of climate change. 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

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expand range and timing of diazotrophic cyanobacteria blooms in the future. Higher temperatures will also increase stratification, flat the mixed layer, and suppress the upwelling of nitrate (Doney, 2006), further promoting the growth of diazotrophic organisms such as *Nodularia*.

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 efficient compared to other phytoplankton in the Baltic Sea (Vahtera et al., 2010) the reduction in inorganic P 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). If climate change promotes cyanobacterial blooms, these toxins might spread further in the ocean and in the food-web and eventually effect humans.

5 Conclusions

In conclusion, our results suggest that *Nodularia spumigena* can grow faster under elevated pCO_2 by enhancing the recycling of organic matter to acquire nutrients. To predict the effect of changing pCO_2 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-discuss.net/9/5109/2012/bgd-9-5109-2012-supplement.zip.

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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 specific alkaline phosphatase activity (sAPA) gives the hydrolysis rate of the substrate in nmol per µg chlorophyll-*a* 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}	$\frac{\text{nmol}}{\text{l} \times \text{h}}$	76.7 ± 5.8	93.6 ± 4.4	62.9 ± 2.6
	sAPA	$\frac{\text{nmol}}{\mu\text{g chl } a \times \text{h}}$	101.6 ± 14.8	108.2 ± 18.2	88.5 ± 6.5
	K_m	µM	0.6 ± 0.4	0.9 ± 0.3	0.7 ± 0.2
Day 9	V_{max}	$\frac{\text{nmol}}{\text{l} \times \text{h}}$	419.5 ± 17.0	693.2 ± 36.8	958.2 ± 54.5
	sAPA	$\frac{\text{nmol}}{\mu\text{g chl } a \times \text{h}}$	90.4 ± 17.2	135.9 ± 25.2	132.0 ± 5.1
	K_m	µM	2.2 ± 0.5	3.9 ± 1.0	4.9 ± 1.2

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Table 2. Correlation of alkaline phosphatase activity (APA) with biomass of filamentous cyanobacteria (POC, chl *a*), PO_4, DOP, pCO_2, pH, N_2 fixation, and bacterial cell counts ($n = 36$, except for PO_4 ($n = 35$) and N_2 fixation ($n = 32$); R = correlation coefficient). Correlation level is marked as * for low significance ($0.03 < |R| < 0.7$, $p < 0.05$) and ** for strong significance ($|R| > 0.7$, $p < 0.05$), nc: for no correlation.

Parameter	Correlation coefficient R	Correlation
AP activity vs.		
PO_4	−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

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Table 3. Glucosidases and leucine aminopeptidase activities at different pCO_2 (V_{max} in nmol per litre and hour and standard deviation; $n = 3$) on day 15 (= day of highest activities).

Enzyme			pCO_2 treatment low	medium	high
α-glucosidase	total	$\frac{\text{nmol}}{\text{l} \times \text{h}}$	15.6 ± 2.8	15.5 ± 2.7	18.8 ± 7.9
	per cell	$\frac{\text{amol}}{\text{l} \times \text{h}}$	28.5 ± 19.9	77.6 ± 22.6	46.9 ± 51.2
β-glucosidase	total	$\frac{\text{nmol}}{\text{l} \times \text{h}}$	18.7 ± 2.4	16.1 ± 2.9	18.1 ± 7.1
	per cell	$\frac{\text{amol}}{\text{l} \times \text{h}}$	33.0 ± 21.1	81.4 ± 14.7	42.9 ± 44.7
Leucine aminopeptidase	total	$\frac{\text{nmol}}{\text{l} \times \text{h}}$	268.0 ± 133.8	375.6 ± 60.7	244.6 ± 58.2

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Table 4. Correlation of leucine aminopeptidase activity with biomass of filamentous cyanobacteria (POC, chl *a*), dissolved inorganic and organic N, 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 ($0.03 < |R| < 0.7$, $p < 0.05$) and ** for strong significance ($|R| > 0.7$, $p < 0.05$), 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

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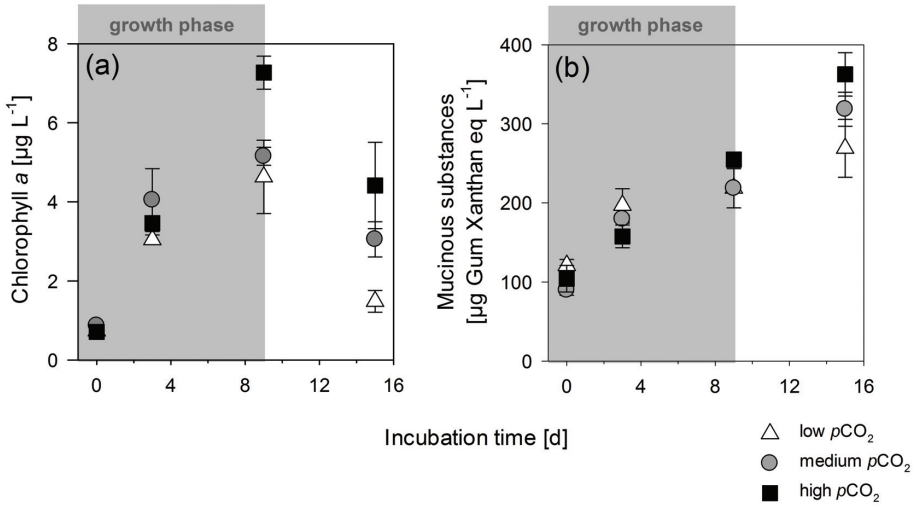

Fig. 1. Changes in chlorophyll-*a* concentration **(a)** and mucinous substance concentration **(b)** over time at low (triangle), medium (circle) and high (squares) pCO_2.

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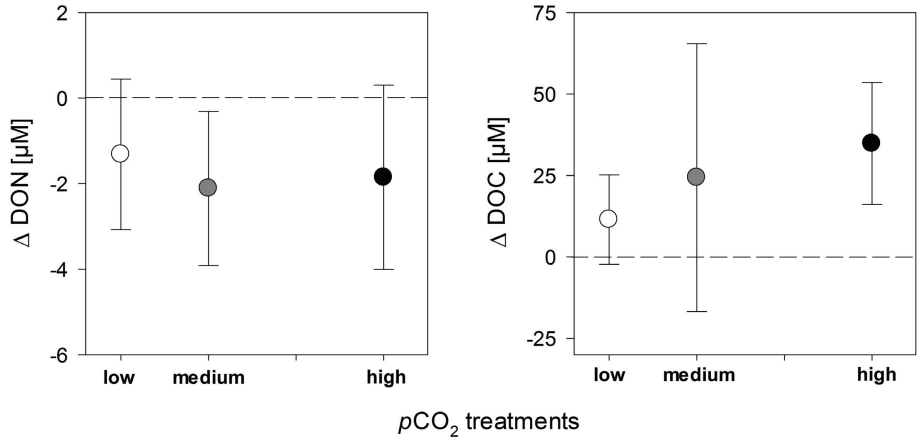

Fig. 2. Changes in the DON and DOC concentrations during the growth phase from day 0 to day 9. Values are not significantly different from each other (*t*-test, $p > 0.05$, $n = 12$).

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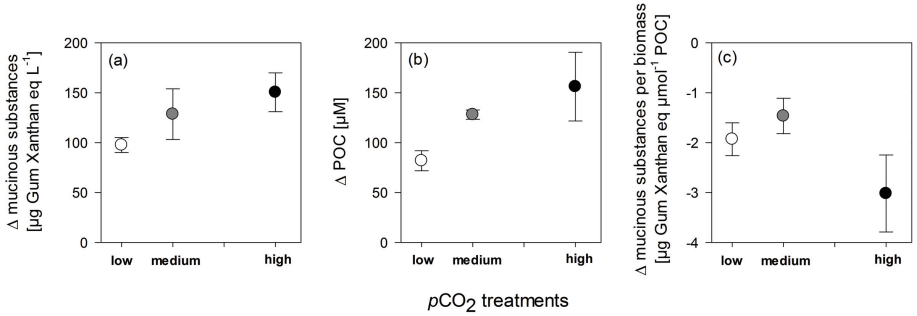

Fig. 3. Comparison of biomass production and accumulation of mucinous substances during the growth phase (day 0 to day 9). Symbols display the mean of three replicates with standard deviation.

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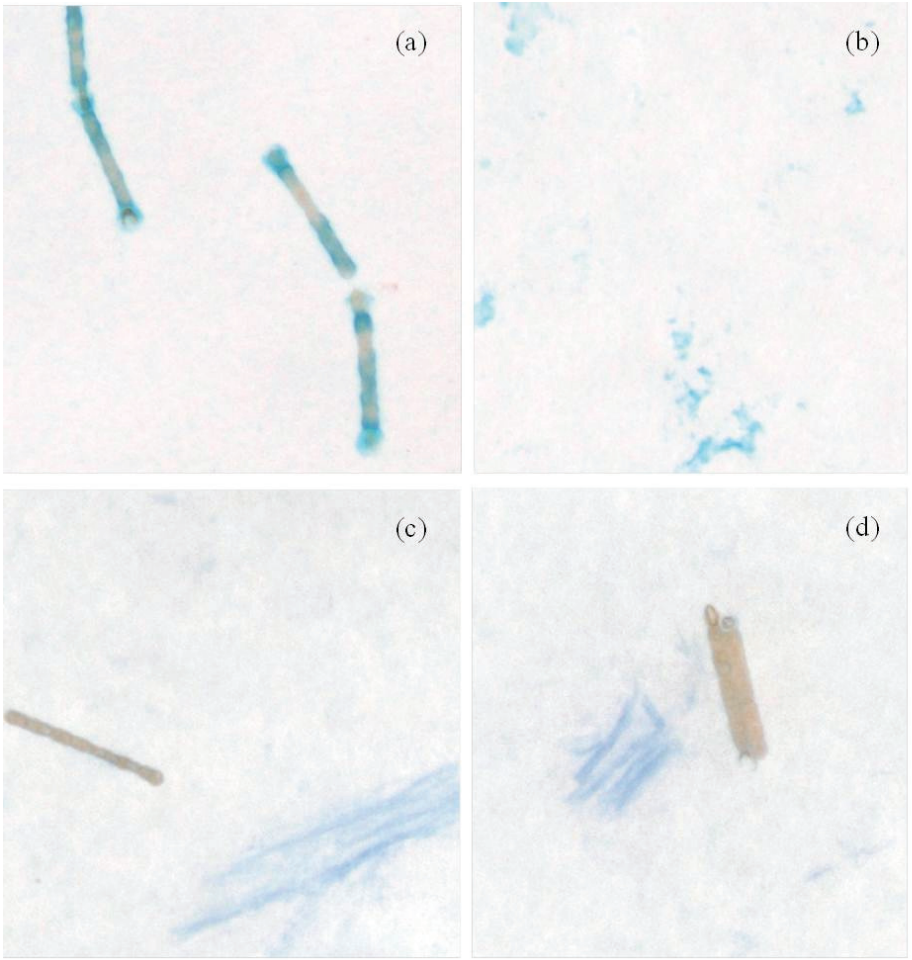

Fig. 4. Microscopic view of *Nodularia spumigena* cultures covered with a mucus coating **(a)**, stained TEP **(b)** and CSP **(c, d)**.

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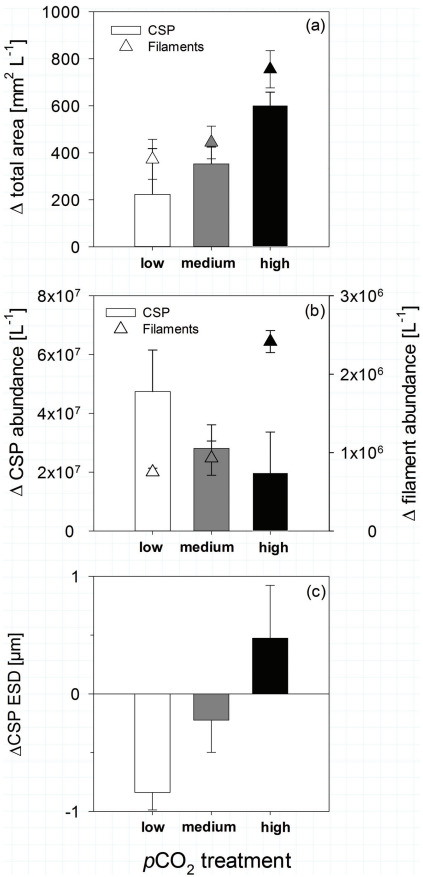

Fig. 5. Variation of the filaments and CSP during the growth phase (day 0 to day 9) in the low (white), medium (grey) and high (black) pCO_2 treatments: **(a)** total area of the filaments and CSP; **(b)** filament and CSP abundance; **(c)** equivalent spherical diameter (ESD) of CSP.

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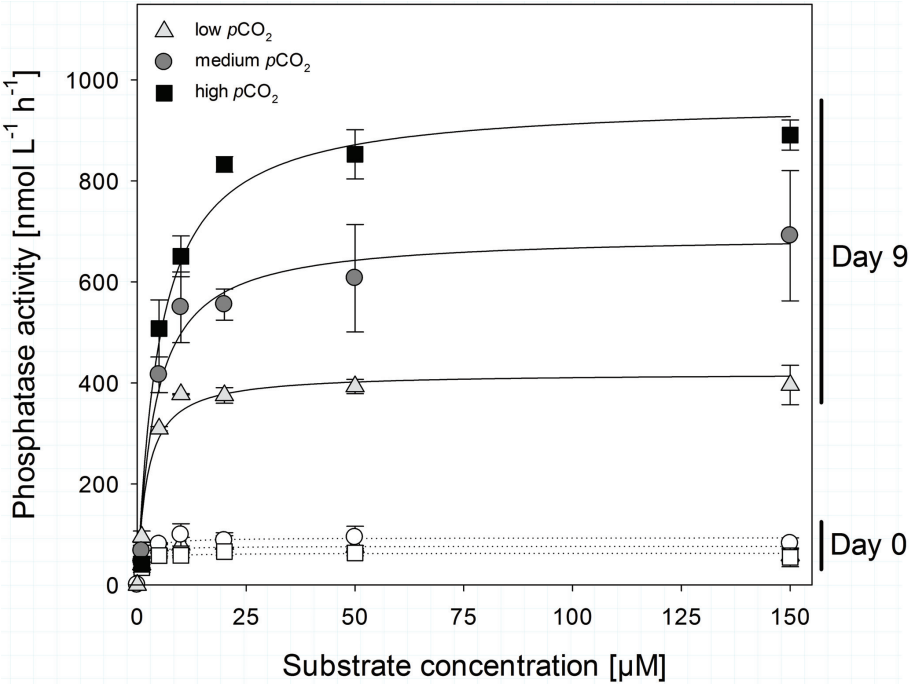

Fig. 6. Total alkaline phosphatase activity at day 0 and day 9 of low (triangle), medium (circle) and high (square) pCO_2 treatments.

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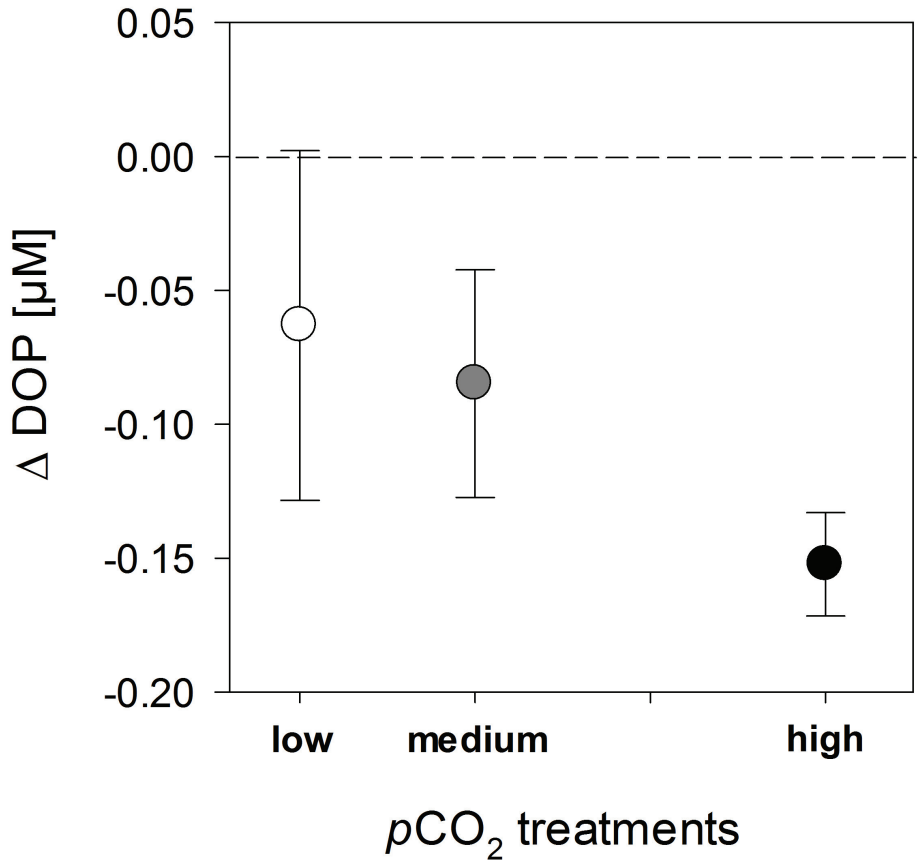

Fig. 7. Changes in the DOP concentration from day 0 to day 9. Low and high pCO_2 treatments ($p = 0.097$) as well as medium and high pCO_2 treatments ($p = 0.066$) are not significantly different.

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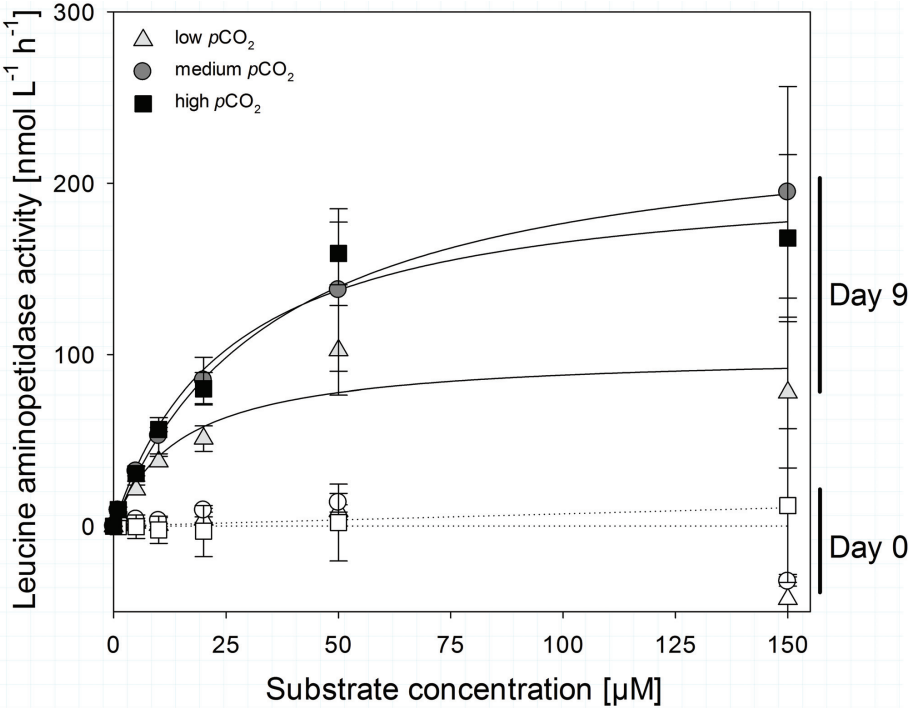

Fig. 8. Aminopeptidase activities on day 0 and day 9 of low (triangle), medium (circle) and high (square) pCO_2 treatments (n = 3).

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