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Response of *Nodularia spumigena* to pCO_2 – Part I: Growth, production and nitrogen cycling

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Abstract

Heterocystous cyanobacteria of the genus *Nodularia* form extensive blooms in the Baltic Sea contributing substantially to the total annual primary production. Moreover, they dispense a large fraction of new nitrogen to the ecosystem, when inorganic nitrogen concentration in summer is low. Thus, it is of great ecological importance to know how *Nodularia* will react to future environmental changes, in particular to increasing carbon dioxide (CO_2) concentrations and what consequences there might arise for cycling of organic matter in the ocean. Here, we determined carbon (C) and dinitrogen (N_2) fixation rates, growth, elemental stoichiometry of particulate organic matter and nitrogen turnover during batch growth of the heterocystous cyanobacterium *Nodularia spumigena* under glacial (180 ppm), present (380 ppm), and future (780 ppm) CO_2 concentrations. Our results demonstrate an overall stimulating effect of rising pCO_2 on C and N_2 fixation, as well as on cell growth. An increase in pCO_2 resulted in an elevation in growth rate, C and N_2 fixation by 23 %, 36 % and 25 %, respectively (180 ppm vs. 380 ppm) and by 27 %, 2 % and 4 %, respectively (380 ppm vs. 780 ppm). Additionally, elevation in the carbon and nitrogen to phosphorus quota of the particulate biomass formed (POC:POP and PON:POP) was observed at high pCO_2. Our findings suggest that rising pCO_2 stimulates the growth of heterocystous diazotrophic cyanobacteria, in a similar way as reported for non-heterocystous diazotrophs. Implications for biogeochemical cycling and food web dynamics, as well as ecological and socio-economical aspects in the Baltic Sea are discussed.

1 Introduction

In summer, the heterocystous diazotrophic cyanobacteria of the genus *Nodularia* form extensive blooms in the open Baltic Sea with more than 200 mg m^{-3} wet weight (Wasmund, 1997), along with cyanobacteria of the genus *Aphanizomenon*. These blooms are usually promoted by low nitrogen-to-phosphorus ratios in the surface waters (e.g.

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Niemistö et al., 1989; Nausch et al., 2008; Raateoja et al., 2011), exhibiting an average annually primary production rate of ~21 mol C m^{-2} yr^{-1} in the Baltic Proper (Wasmund et al., 2001b). The capacity of community N_2 fixation in the Baltic Sea is comparable to nitrogen inputs from the land and atmosphere (e.g. Larsson et al., 2001; Wasmund et al., 2001b, 2005b). Annual N_2 fixation rates during a moderate bloom in the Baltic Proper were averaged to 101–263 mmol N m^{-2} yr^{-1} (Wasmund et al., 2001a).

A significant fraction of the newly fixed nitrogen can be directly released by cyanobacteria, thereby dispensing 35 to 80 % into the surrounding environment (Glibert and Bronk, 1994; Wannicke et al., 2009; Ploug et al., 2011). This new nitrogen can be transferred to lower food web levels via the dissolved fraction (Ohlendieck et al., 2000) and to higher trophic levels by grazing directly on cyanobacteria or indirectly via the microbial loop (Engström-Ost et al., 2011). The extra load of nitrogen thus increases overall ecosystem productivity and meets 20 to 90 % of the nitrogen requirements for community primary production during summer blooms (Sörensson and Sahlsten, 1987; Larsson et al., 2001; Wasmund et al., 2005a). A large part of the biomass formed by cyanobacteria is lost from the upper mixed layer through aggregation and sedimentation and accounts for a considerable proportion of the seasonal sinking flux (Lignell et al., 1993; Heiskanen and Leppänen, 1995).

To date, it is not well understood and barely investigated, how future changes in climate caused by anthropogenic elevation of atmospheric CO_2 concentration will affect *Nodularia* performance and their potential to alter biogeochemical fluxes. The atmospheric partial pressure of CO_2 (pCO_2) has increased by roughly one-third at present day compared to the preindustrial times, accounting for the highest levels since approximately half a million years (e.g. Lüthi et al., 2008). With atmospheric CO_2 dissolving in seawater, it is expected that current pCO_2 in the oceans will nearly double to 780 µatm by 2100, and lower the ocean's pH by about 0.35 units (IPCC, 2007), assuming that emissions will carry on at the present rate. This has severe implications for marine phytoplankton, as they appear to directly respond to increasing pCO_2 by altering their physiology (e.g. Riebesell et al., 2007), relative abundance (e.g. Tortell

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et al., 2002), and biogeography (e.g. Boyd and Doney, 2002). Additionally, unicellular marine cyanobacteria such as *Synechococcus* and *Prochlorococcus* can show species- specific responses to increasing pCO_2 (e.g. Fu et al., 2008). Several studies demonstrate that elevated pCO_2 supports C and N_2 fixation, as well as growth rates in *Trichodesmium* (Hutchins et al., 2007; Levitan et al., 2007; Fu et al., 2008; Barcelos e Ramos et al., 2007). It has been hypothesized that these trends are facilitated by changes in activity of the carbon concentrating mechanism (CCM) and modified protein activity (Levitan et al., 2010a, b; Kranz et al., 2011) of the enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (RUBISCO) resulting in a decrease of energy and nutrient demand of the cell at high pCO_2. The enzyme RUBISCO has a naturally low affinity to carbon. Subsequently, energy saved can be relocated to other metabolic processes such as N_2 fixation. But experimental data so far are not able to prove this hypothesis on a gene expression level and no publication is available to verify this notion for heterocystous cyanobacteria.

Additionally, it is largely unknown how heterocystous cyanobacteria respond to in pCO_2 in general, Czerny et al. (2009) directly addressed the effects of different pCO_2 conditions on growth and C fixation of the genus *Nodularia* and observed an overall detrimental effect of rising pCO_2 on the cells associated with a decrease in growth and production. They suggested that this pattern could be typical for heterocystous cyanobacteria compared to non-heterocystous cyanobacteria of the genus *Trichodesmium* and potentially relate to physiological and structural dissimilarities of both cyanobacteria groups. Even less information is available on the coupling of fluxes of carbon, nitrogen and phosphorus in relation to pCO_2 in heterocystous cyanobacteria.

Therefore, the purpose 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. Cultures of Baltic Sea *Nodularia spumigen*a isolates were grown in a batch mode across three different pCO_2 levels, simulating glacial (180 ppm), present day values (380 ppm) and values projected for the year 2100 (780 ppm). Here, we present data on growth and production parameters, as well as N_2 fixation and

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nitrogen turnover in response to increasing pCO_2. Carbon cycling and extracellular enzyme activities as well as phosphorous cycling and utilization of dissolved organic phosphorous (DOP) will be presented in two companion publications.

2 Material and methods

2.1 Culture condition and design of the batch culture experiment

The experimental set-up is illustrated in Fig. 1. The heterocystic cyanobacterium *N. spumigena* was isolated by L. Stal and coworkers (NIOO) from the Baltic Sea and maintained at the Leibniz Institute for Baltic Sea Research in batch cultures in f/2 medium free of any combined N compounds. Parent cultures were transferred weekly to aged Baltic Sea water 3 weeks prior to the start of the experiment. *N. spumigena* was cultured at 15 °C in a walk-in incubation chamber equipped with a cycle of 16:8 h light: dark (cool, white fluorescent lighting, 100 µmol photons m^{-2} s^{-1}). One week before the start of the acclimation period, parent cultures were removed from the walk-in incubation chamber to a climate controlled room. Here temperature was increased to 23 °C (representing typical summer temperatures at the Baltic Sea water surface) and light supply doubled to 200 µmol photons m^{-2} s^{-1} (light: dark cycle of 16:8 h, cool, white fluorescent lighting). Parent cultures were axenic when starting the experiments (cells counts below blank value of 1000 cells l^{-1}). Overall bacterial biomass during the course of the experiments (18 days = 15 days experiment + 3 days acclimation) never exceeded 1 % of cyanobacterial biomass. Bacterial abundance and biomass were monitored by flow cytometry (Gasol and Giorgio, 2000). Cultures were routinely mixed by manually shaking to prevent adhesion of cyanobacteria to the walls of the culture vessels.

In preparation of the experiment 1000 l of Baltic seawater were collected in open waters of the Baltic Sea (54.22749° N, 12.1748° E, salinity of 9.1 psu), four months before the start of the experiment. From this, cell free, aged seawater was prepared,

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whereby inorganic nutrient were removed by phytoplankton and bacterial growth, and sterilisation was achieved by UV light treatment and 0.2 µm filtration under a clean bench. Concentrations of inorganic nutrients in this seawater (DIN and DIP) were below the detection limit.

Three pCO_2 treatments were selected according to the guide to best practice for ocean acidification research (Riebesell et al., 2010): 180 ppm (representing glacial conditions), 380 ppm (representing present day) and 780 ppm (representing year 2100 conditions), (Boer et al., 2000). Adjustment of pCO_2 was done by aeration of each bottle separately with premixed gases (Linde gas). During the duration of the experiment (18 d), aeration took place in the early afternoon (02:00 p.m. LT) for one hour per day in order to minimize the formation of aggregates and to avoid high turbulence that could potentially harm the integrity of the cyanobacteria filaments.

Acclimation of the *N. spumigena* parent culture started three days before the beginning of the experiment. Therefore, the parent culture was separated into three pre-cultures, one litre for each of the CO_2 treatment, and acclimated to the target pCO_2 by aeration with the target gas mixture, as described above.

After three days of acclimation, chlorophyll-*a* concentrations were determined in the three pre- cultures in order to inoculate the same quantity to each replicate bottle. Four sampling time points were selected for the three pCO_2 treatments (time 0, +3, +9 and +15 days, Fig. 1) with three replicate bottles harvested at each time point. Each bottle contained 10 l of aged and sterile filtered seawater that had been aerated with premixed gases for three days in parallel with the pre-cultures. After inoculation with *N. spumigena* (volume equal to a final concentration of chlorophyll-*a* of 0.8 µg l^{-1}), each of the 36 bottles was amended with phosphate to 0.35 µmol l^{-1} at time 0 and to 0.35 µmol l^{-1} at day 3. Subsequent to inoculation and phosphate addition, three bottles per CO_2 treatment were sampled at time 0 and then at day 3, 9 and 15. One replicate bottle of the 180 ppm pCO_2 treatment at day 9 was omitted in the data compilation, due to inaccurate inoculation with PO_4.

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2.2 Analytical methods

2.2.1 Carbonate chemistry

pH was measured with an electrode (Knick Mikroprozessor pH Meter 761 with Typ SE 100 glass electrode), calibrated directly before measurement with standard NBS buffer. Values of pH are given relative to the NBS scale.

Total carbon (C_T) was analysed directly after sampling using the colorimetric SOMMA system according to Johnson et al. (1993). The system was calibrated with carbon reference material provided by A. Dickson (University of California, San Diego) and yielded a precision of about ±2 µmol kg^{-1}.

Total alkalinity and pCO_2 were calculated using CO2SYS (Lewis et al., 1998) parallel to C_T, pH, salinity and temperature.

2.2.2 Nutrient and chlorophyll-*a* analysis

Dissolved inorganic nutrients (NH_4^+, NO_3^- and PO_4^{3-}) were determined colorimetrically from 60 ml filtered subsamples (combusted GF/F) using a spectrophotometer U 2000 (Hitachi-Europe GmbH, Krefeld, Germany) according to Grasshoff et al. (1983). The detection limits were 0.02 µmol l^{-1} for DIP, 0.05 µmol l^{-1} for ammonium and 0.05 µmol l^{-1} for NO_3. A subsample of 100 ml was filtered onto Whatman GF/F filters for chlorophyll-*a* analysis, immediately after sampling. Filters were stored in liquid nitrogen or at −80 °C and were extracted with 96 % ethanol for at least 3 h. Chlorophyll-*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). Chlorophyll-*a* concentrations were calculated according to the method of Jeffrey and Welschmeyer (1997).

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2.2.3 *Nodularia* filament and bacteria cell counts

Subsamples of 50 ml were taken for phytoplankton analysis (preserved with acetic Lugol's (KI/I2) solution to 1 % fixation) and counted using an inverted microscope (Leica) (Utermöhl, 1958) at 100 × magnification. Cell length and diameter were measured using a micrometer eyepiece and converted to biovolume assuming the geometrical approximation of a cylinder.

Bacteria were counted using a flow cytometer (Facs Calibur, Becton Dickinson) following the method of Gasol and del Giorgio (2000). Four millimeter samples were preserved with 100 µl formaldehyde (1 % v/v final concentration), shock frozen in liquid nitrogen and stored at −70 °C until measurement. A stock solution of SYBR GREEN (Molecular Probes) was prepared by mixing of 1 µl dye with 49 µl DMSO. Three microliter 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 darkness. Cell counting was done at a medium flow rate and calculations were performed using the software program "Cell Quest Pro". Mean abundance of heterotrophic bacteria was $4.45 \pm 2.28 \times 10^5$ cells l^{-1} (180 ppm), $2.38 \pm 2.09 \times 10^5$ cells l^{-1} (380 ppm) and $4.80 \pm 2.82 \times 10^5$ cells l^{-1} (780 ppm). There was no significant increase in bacterial abundance indicating absence of bacterial growth.

2.2.4 Dissolved organic matter (DON, DOC, DOP)

For analysis of dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) subsamples were filtered through pre-combusted GF/F filters, collected in 20 ml pre-combusted (8 h, 500 °C) glass ampoules, acidified with 80 µl of 85 % phosphoric acid and stored frozen at 2–5 °C. TDN and DOC concentrations were determined simultaneously by high temperature catalytic oxidation with a Shimadzu TOC-VCSH analyser. In the auto sampler 18 ml of sample volume plus 9 ml of ultrapure (Type 1) water (in pre-combusted vials) were acidified with 50 µl HCl (1 M) and sparged with oxygen (150 ml min^{-1}) for 6 min to remove all inorganic C. 100 µl sample volume was injected

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directly on the catalyst (heated to 720 °C). Detection of the generated CO_2 was performed with an infrared detector. Final DOC concentrations were average values of quadruplicate measurements. If the coefficient of variation exceeded 0.1 %, up to 4 additional analyses were performed and outliers were eliminated. Total N was quantified by a chemiluminescence detector (gas flow oxygen: 0.6 l min^{-1}). After every 8th sample, one standard for quality control and one blank was measured. Values of TDN were corrected for nitrate, and ammonium, and thereafter referred to as DON.

Subsamples (40 ml) for the determination of total (TP) and dissolved phosphorus (DP) were stored at −20 °C until processing either unfiltered (for TP) or filtered through pre-combusted (450 °C, 4 h) Whatman GF/F filters (for DP). 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 subsequent DIP determination was done using a 10 cm-cuvette reducing the detection limit to 0.01 µM. Dissolved organic phosphorus (DOP) was calculated as the difference between dissolved phosphorus (DP) and dissolved inorganic phosphorous (DIP), detected as described above.

2.2.5 Particulate organic matter analysis (PON, POC, POP)

Stable N and C isotope ratios (δ^{15}N-PON, δ^{13}C-POC), as well as PON and POC concentration were measured by means of flash combustion in a Carlo Erba EA 1108 at 1020 °C and a Thermo Finnigan Delta S mass-spectrometer. Filters containing particle samples were trimmed, sectioned and then loaded into tin capsules and pelletised for isotopic analysis. Particulate organic phosphorus (POP) was calculated as the difference between total and dissolved phosphorus.

2.2.6 Isotopic analysis and rates measurements (primary production, N_2 fixation)

The stable N and C isotope ratios measured for each sample were corrected for values obtained from standards with defined N and C isotopic compositions (International

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Atomic Energy Agency IAEA: IAEA-N1, IAEA-N2, NBS 22 and IAEA-CH-6) by means of mass balance. Values are reported relative to atmospheric N_2 ($\delta^{15}N$) and VPDB ($\delta^{13}C$- Vienna Peedee belemnite). The analytical precision for both stable isotope ratios was ±0.2 ‰. Calibration material for C and N analysis was acetanilide (Merck). N_2 fixation activity was measured using the ^{15}N-N_2 assay, C fixation using the ^{13}C-$NaHCO_3$ assay. Tracer incubations were terminated by gentle vacuum filtration (<200 mbar) through pre-combusted GF/F filters. These filters were dried at 60 °C and stored for isotopic analysis. Rates were calculated using the approach of Montoya et al. (1996). Incubation time for rate measurements was 6 h, guaranteeing a sufficient dissolution of the ^{15}N gas in the incubation bottle (method consideration; Mohr et al., 2010). To compare these results to literature data and to relate them to cyanobacteria biomass, rates were normalized to filament number.

2.3 Nitrogen and carbon turnover

A model of daily C and N flow was calculated from averaged production rates, C and N_2 fixation, as well as PON and POC build up of day 0 to day 9. Total nitrogen (TN) was calculated as the sum of DIN, DON and PON.

Exudation of TDN and DOC was determined from the differences in concentration of subsequent sampling days. Respiration was estimated by determining the differences between C fixation and build up of POC plus DOC exudation on a daily basis.

2.4 Statistical analysis

Statistical analyses were done using the software SPSS 9.0 and Sigma Plot 10. Differences between pCO_2 treatments were identified by post hoc standard least square contrast analyses after analysis of covariance (ANCOVA) with time as the covariate and pCO_2 as the nominal predictor. Dependencies of growth and production parameters from other environmental parameters were tested using Pearson's correlation "stepwise" multiple regression analysis. Prior to ANCOVA and correlation analysis,

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data were tested for normality and homogeneity of variances using Wilk-Shapiro and Levene's tests. Linear regression analysis was applied to calculate growth rates from changes in natural logarithm transformed filament/cell numbers, PON, POC, as well as chlorophyll-*a* values. Comparison of mean concentrations was done using Student's t-test.

3 Results

3.1 Carbonate chemistry

Throughout the study, the pCO_2 treatments were different with respect to pH and total carbon (C_T), as well as calculated total alkalinity (A_T) and pCO_2 (Table 1). The pCO_2 treatments differed significantly in pH and C_T between 380 ppm and 780 ppm, as well as between 180 ppm and 780 ppm (ANCOVA, $p < 0.001$, $n = 12$, Table 2). The calculated pCO_2 was significantly different between all three pCO_2 set-ups (ANCOVA, $p = 0.001$ and $p < 0.001$, $n = 12$, Table 2).

3.2 Inorganic nutrients

Dissolved inorganic phosphate was depleted in all treatments after three days of incubation (Table 3). DIP amended on day 3 was again below the detection limit at day 9. Throughout the experiment, mean concentration of dissolved inorganic nitrogen ($DIN = NO_3^- + NO_2$) was $0.26 \pm 0.1\ \mu mol\ l^{-1}$ in the 180 ppm pCO_2 treatment, $0.13 \pm 0.1\ \mu mol\ l^{-1}$ in the 380 ppm pCO_2 treatment and $0.1 \pm 0.1\ \mu mol\ l^{-1}$ in the 780 ppm pCO_2 treatment (Table 3), whereas ammonium was not detectable. There were no significant differences in concentrations of inorganic nutrients between pCO_2 treatments (Table 2). PO_4^{3-} correlated significantly with DON and DOP ($R^2 = 0.599$ and 0.359, $p < 0.05$, $n = 12$). Due to the uptake of nutrients during cell growth, and inverse relationship were observed between DIP and POC, PON and POP, respectively ($R^2 = -0.569$, -0.596 and -0.622, $p < 0.001$, $n = 12$).

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3.3 Dissolved organic matter (DOM)

Overall averages of DOM concentrations were in the 180 ppm pCO_2 treatment $303 \pm 26\,\mu mol\,l^{-1}$ for DOC, $15 \pm 1.0\,\mu mol\,l^{-1}$ for DON, and $0.3 \pm 0.1\,\mu mol\,l^{-1}$ for DOP. In the 380 ppm pCO_2 treatment concentrations were: $309 \pm 21\,\mu mol\,l^{-1}$ for DOC, $16 \pm 1.3\,\mu mol\,l^{-1}$ for DON and $0.27 \pm 0.1\,\mu mol\,l^{-1}$ DOP. Concentration in the 780 ppm pCO_2 treatment was: $313 \pm 36\,\mu mol\,l^{-1}$ for DOC, $17 \pm 1.2\,\mu mol\,l^{-1}$ for DON and $0.27 \pm 0.08\,\mu mol\,l^{-1}$ for DOP.

In the course of the first 3 days of experiment DOC concentration decreased in the 180 ppm and 780 ppm pCO_2 treatment by 35 and $33\,\mu mol\,l^{-1}$, respectively, while it increased in the 380 ppm pCO_2 treatment by $2\,\mu mol\,l^{-1}$. In the same period, DON concentration was elevated in all treatments by $17 \pm 1\,\mu mol\,l^{-1}$. Henceforward, till the end of the experiment at day 15, concentrations of DOC increased by $24\,\mu mol\,l^{-1}$ in the 180 ppm pCO_2 treatment, by $13\,\mu mol\,l^{-1}$ in the 380 ppm pCO_2 treatment and by $5\,\mu mol\,l^{-1}$ in the 780 ppm pCO_2 treatment, respectively, while DON concentration decreased again by $0.3\,\mu mol\,l^{-1}$, $0.11\,\mu mol\,l^{-1}$ and $0.9\,\mu mol\,l^{-1}$, respectively. Nevertheless, calculated differences in concentration are of the same magnitude as standard deviation of the single measurements and have to be considered carefully.

Differences in DOM between the pCO_2 treatments were not statistically significant, except for DON concentrations, which were significantly lower in the 180 ppm compared to the 780 ppm pCO_2 treatment ($p = 0.045$, $n = 12$, Table 2), indicating a higher accumulation of DON at high pCO_2. DOC correlated significantly negative with N_2 fixation rates ($R^2 = -0.38$, $p < 0.05$, $n = 12$) and positive with PON ($R^2 = 0.356$, $p < 0.05$, $n = 12$). DOP showed a significantly negative correlation with cyanobacterial abundance, POC, PON and POP ($R^2 = -0.493$, -0.495, -0.574 and -0.844, $p < 0.05$ and <0.01, $n = 12$) and a positive one with PO_4^{3-} ($R^2 = 0.359$, $p < 0.05$, $n = 12$). DON showed a significantly negative correlation with PON, POP and pH ($R^2 = -0.351$, -0.333 and -0.619, $p < 0.05$ and $p < 0.01$, $n = 12$) and positive ones with C fixation,

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PO_4^{3-}, pCO_2 and C_T (R^2 = 0.537, 0.622 and 0.603, $p < 0.01$, $n = 12$). DOM stoichiometry did not differ significantly between the treatments. Mean values for DOC:DON ratios were 20 ± 3 (180 ppm), 19 ± 2 (380 ppm) and 19 ± 2 (780 ppm). Mean DOC:DOP ratios were 1094 ± 383 (180 ppm), 1249 ± 421 (380 ppm) and 1243 ± 378 (780 ppm). Ratios of DON:DOP were 57 ± 14 (180 ppm), 64 ± 17 (380 ppm) and 66 ± 19 (780 ppm).

3.4 Responses of *Nodularia*

3.4.1 *Nodularia* abundance

There was a steady increase in the abundance of *Nodularia* filaments in all pCO_2 treatments until day 9 (Fig. 2). Abundance remained static or declined slightly thereafter. A Turkey's pair wise comparison revealed statistically significant differences with treatments 180 ppm vs. 780 ppm ($p < 0.001$, $n = 12$) and 380 ppm vs. 780 ppm ($p = 0.007$, $n = 12$, Table 2). *Nodularia* abundance correlated significantly positive with chlorophyll-*a*, POC, PON and POP (R^2 = 074, 0.83, 0.88 and 0.88, $p < 0.01$, $n = 12$). Thus, accumulation of *Nodularia* filaments significantly increased at high pCO_2.

Mean filament length in the 780 ppm pCO_2 treatment increased from 74 ± 28 µm (day 0) to 88 ± 38 µm (day 9) but his trend was not statistically significant. Filament length in the 180 ppm and 380 ppm pCO_2 treatments increased from 67 ± 24 µm to 117 ± 53 µm, and from 71 ± 29 µm to 100 ± 50 µm, respectively. Differences in filament length between treatments were not statistically significant, but filaments were slightly shorter at the highest pCO_2 by ~23 %. During the first three days, there was a significantly higher number of heterocysts per filament in the 780 ppm pCO_2 treatment compared to the 380 ppm pCO_2 treatment ($p = 0.005$, $n = 12$) and the 780 ppm pCO_2 treatment compared to the 180 ppm pCO_2 treatment ($p = 0.0001$, $n = 12$). Henceforward, heterocyst number per filament decreased in all pCO_2 treatment.

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3.4.2 Chlorophyll-*a*

Chlorophyll-*a* increased over time in all pCO_2 treatments (Table 3). ANCOVA and Turkey's post hoc test proved significant differences between pCO_2 treatments: 180 ppm vs. 380 ppm ($p < 0.001$, $n = 12$), 180 ppm vs. 780 ppm ($p < 0.001$, $n = 12$) and 380 ppm vs. 780 ppm ($p = 0.006$, $n = 12$, Table 2) with highest mean chlorophyll-*a* values at 780 ppm ($3.96 \pm 0.42\,\mu g\,l^{-1}$), mid values at 380 ppm ($3.28 \pm 0.72\,\mu g\,l^{-1}$), and lowest values at 180 ppm ($2.28 \pm 0.79\,\mu g\,l^{-1}$).

Chlorophyll-*a* concentration correlated significantly positive with abundance, POC, PON and POP concentration ($R^2 = 0.741$, 0.845, 0.781 and 0.617, $p < 0.01$, $n = 12$).

3.4.3 Concentration and stoichiometry of particulate organic matter (POM)

Concentrations of POC, PON and POP increased in all pCO_2 treatments, but most pronounced at 780 ppm (Fig. 3). POC and PON concentration differed significantly between pCO_2 treatments with highest concentrations being observed at high CO_2 (Table 2). Normalized to filament abundance, however, POC, PON and POP were lowest in the high pCO_2 treatment (Fig. 3). Thereby, differences in POC content per filament were statistically significant for 380 ppm vs. 780 ppm ($p = 0.05$, $n = 12$), but not for all other pCO_2 treatments. PON and POP per filament differed significantly between the pCO_2 treatments 180 ppm and 780 ppm ($p = 0.05$ and $p = 0.01$, $n = 12$) and between 380 ppm and 780 ppm ($p = 0.05$ and $p = 0.01$, respectively, $n = 12$).

Box-plots of POM elemental composition demonstrate an elevation in all treatments relative to Redfield ratios for POC:POP and PON:POP, but near Redfield stoichiometry for POC:PON (Fig. 4). Differences in stoichiometry between the single treatments were significant for all ratios comparing pCO_2 treatments: 180 ppm vs. 380 ppm and 180 ppm vs. 780 ppm ($p < 0.001$, $n = 12$, Table 2).

A significantly negative correlation of elemental composition (POC:PON, POC:POP and PON:POP) occurred on the one hand with pCO_2 ($R^2 = -0.552$, -0.556 and -0.529, respectively, $p < 0.01$, $n = 12$). On the other hand, this relationship

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also appeared between elemental composition and C fixation rates per filament ($R^2 = -0.664$, -0.675 and -0.679, respectively, $p < 0.01$, $n = 12$) and PO_4^{3-} ($R^2 = -0633$, -0.653 and -0.634, respectively, $p < 0.01$, $n = 12$). This might imply a more balanced incorporation of C, N and P at higher pCO_2.

POC:PON, POC:POP and PON:POP correlated significantly positive with abundance ($R^2 = 0.751$, 0.795 and 0.789, respectively, $p < 0.01$, $n = 12$), chlorophyll-*a* ($R^2 = 0.823$, 0.883 and 0.829, respectively, $p < 0.01$, $n = 12$) and pH ($R^2 = 0.529$, 0.531 and 0.501, respectively, $p < 0.01$, $n = 12$).

3.4.4 Growth rates

Growth rates calculated from changes in POC and PON were lower than growth rates derived from abundance and chlorophyll-*a* in the 180 ppm pCO_2 treatment, while they were equal in the 380 ppm and 780 ppm pCO_2 treatments (Fig. 5). Compiled growth rates based on all parameters were significantly different ($p < 0.05$, $n = 12$) between the pCO_2 treatments with highest growth rate at 780 ppm ($0.212 \pm 0.018\ d^{-1}$, Table 2).

3.4.5 C and N_2 fixation

C and N_2 fixation rates decreased with incubation time in all pCO_2 treatments (Fig. 6). Nevertheless, statistically significant differences between pCO_2 treatments according to ANCOVA and Turkey's post hoc test were observed for volume specific N_2 fixation rates (380 ppm vs. 780 ppm, $p = 0.045$, $n = 12$, Table 2) and for volume specific C fixation rates (180 ppm vs. the 380, $p < 0.001$, $n = 12$, and 180 ppm vs. 780 ppm, $p = 0.001$, $n = 12$, Table 2). C and N_2 fixation rates were directly related to pCO_2 ($R^2 = 0.66$ and 0.82, $p < 0.05$, $n = 12$). Ratios of C_{fixed}:N_{fixed} were higher than the Redfield ratio and yielded maximum values at 380 ppm (C:N = 16:1) and the lowest at 780 ppm (C:N = 9.6) (Fig. 4). Differences between the pCO_2 treatments were statistically significant according to Student's t-test for 180 ppm vs. 380 ppm ($p = 0.016$, $n = 12$) and 380 ppm vs. 780 ppm ($p = 0.001$, $n = 12$).

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3.5 Nitrogen and carbon turnover

In our model of nitrogen turnover observed in *Nodularia* during the first 9 days (Fig. 7a–c) total nitrogen (TN) increased on a daily basis, attributed to the fixation and incorporation of new N_2 into PON in all treatments. Nevertheless, the increase of PON was highest in the 780 ppm pCO_2 treatment accounting for 3 µmol N l^{-1} d^{-1} compared to 1.2 µmol N l^{-1} d^{-1} in the 180 ppm treatment and 2.5 µmol N l^{-1} d^{-1} in the 380 ppm pCO_2 treatment, respectively. On a daily basis, N_2 fixation exceeded the build up of PON observed in all pCO_2 treatments (Fig. 7a–c). No exudation of dissolved nitrogen compounds (DON and DIN) was detected. Instead, uptake of dissolved nitrogenous compounds (TDN) occurred, which explained 8 % of PON build up in the 180 ppm and 380 ppm pCO_2 treatment and 6 % in the 780 ppm pCO_2 treatment.

Carbon turnover within the first 9 days of the experiment (Fig. 7d–f) revealed only minor differences between the 380 ppm and 780 ppm pCO_2 treatments, while the 180 ppm treatment differed greatly from the other two pCO_2 treatments. Calculated exudation of DOC in the 180 ppm set-up was negative, indicating uptake of DOC integrated over a daily cycle. Respiration as a percentage of gross primary production (C fixation plus DOC exudation) decreased with increasing pCO_2 from 65 % in the 180 ppm treatment to 59 % and 54 % in the 380 and 780 ppm treatment, respectively, suggesting higher cyanobacteria growth efficiency at higher pCO_2.

4 Discussion

4.1 Growth and production under different pCO_2 conditions

In this study we assessed the response of *Nodularia spumigena* to changes in pCO_2 (180, 380 and 780 ppm). Growth rates were highest in the future pCO_2 scenario (780 ppm) being elevated by 27 % relative to the present day set-up (380 ppm) (ANCOVA, $p < 0.001$, $n = 12$) and by even 44 % relative to the glacial pCO_2 treatment

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(180 ppm) (ANCOVA, $p < 0.001$, $n = 12$). Assimilation of C in the high pCO_2 treatment increased by 2 % compared to present day and by even 36 % relative to the glacial scenarios. N_2 fixation was elevated by 4 % and 13 %, respectively. The higher stimulation of nitrogen compared to carbon fixation might lead to elevated input of new N which could promote eutrophication. Elevation in N_2 fixation at the highest pCO_2 was accompanied by a higher number of heterocysts per filament. Nevertheless, heterocyst frequency in all treatments declined over the course of the incubation, possibly because filaments became shorter due to increased cell division. Heterocyst frequency in *Nodularia* (Lindahl et al., 1980) and *Aphanizomenon* (Riddolls, 1985) has been shown to correlate with N_2 fixation rate, suggesting that it could be used as an indicator for N_2 fixation capacity.

Our budget calculation of C suggests a lowering of respiration at high pCO_2, which has to be confirmed in future studies by direct measurements. This might result in an increase in growth efficiency (growth efficiency = net production/net production + respiration) at high pCO_2 by 6.2 % from 180 ppm to 380 ppm and by 5.9 % from 380 to 780 ppm. Comparing the stimulative effect of pCO_2 on the three different rates, we measured the strongest elevation for C fixation when rising pCO_2 from 180 ppm to 380 ppm, and for growth rate when rising pCO_2 from 180 ppm to 780 ppm, as well as from 380 ppm to 780 ppm. This also hints towards decreased growth efficiency at low pCO_2, because at 180 ppm C fixation rates increasing strongest, but was not transferred into growth. Other metabolic processes must have consumed this surplus in energy and metabolites gained by C and N_2 fixation.

In our study growth rates increased with increasing pCO_2 despite DIP limitation indicating a stimulating effect of DIC availability. This notion suggests a co-limitation by C and P in our experimental set-up at glacial and present day pCO_2 conditions, which might be applied for the Baltic Sea in summer, as well. A deficiency in DIP seems to be partly counter-balanced by excess C, which is opposing to the concept of Liebig's law of only one limiting nutrient. This has already been denoted by e.g. Arrigo (2005) and Hutchins et al. (2007).

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The first and only study available so far reporting the response of *Nodularia* growth and primary production to changing pCO_2 conditions was published by Czerny et al. (2009) hypothesizing a detrimental effect of high pCO_2 on *Nodularia* growth.

Both studies investigating *Nodularia* performance, Czerny et al. (2009) and ours, used culture conditions that favoured the formation of single filaments without visible formation of larger aggregates. While Czerny and co-workers continuously moved their incubation bottles using a plankton wheel, we used slight agitation by manually rotating the bottles once a day. On the other hand, the method used to manipulate pCO_2, might partly explain the observed opposing trends. Czerny et al. (2009) adjusted the pH by acid/base manipulation, which changes total alkalinity (TA) at constant dissolved inorganic carbon (DIC). Concentrations of DIC, HCO_3^-, and CO_2^{3-} in their study might have been lower than their actual target values, because seawater pH controls the relative proportion of the carbonate species and induces a lower percentage increase in HCO_3^- compared to a reduction of pH achieved by e.g. aeration or by co-adding carbonate ions along with acid (e.g. $NaHCO_3$) (e.g. Gattuso and Lavigne, 2009). This fact might have dampened a possible positive pCO_2 effect.

In our study, we shifted pH by aeration with premixed gases of the target pCO_2 value at a low flow rate to avoid turbulences and shear stress. Hence, our approach reproduces the projected change in parameters of the carbonate system expected for the year 2100 by altering DIC at constant TA. Additionally, it has been shown that light intensity strongly influences the magnitude of stimulation of growth and production by pCO_2 (e.g. Kranz et al., 2010), with significantly elevated rates at high pCO_2 and light conditions. Light intensity in our experiment was higher by a factor of 2.4 compared to those in Czerny et al. (2009) (200 vs. 85 µmol photons m^{-2} s^{-1}, respectively). Furthermore, Czerny and co-workers hypothesize that the negative effect of high pCO_2 on N_2 fixation and growth occurs, because translocation of amino acids from heterocysts to vegetative cyanobacteria cells was restrained by a reduction in extracellular pH. However, intracellular amino acid translocation is not necessarily directly dependent on the external pH, because ionic exchange between adjacent cells takes place through the

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microplasmodesmata. These are intercellular channels linking the cytoplasms of cells where intracellular pH is kept constant (Mullineaux, 2008; Flores and Herrero, 2010). Therefore, it is unlikely that amino acids will pass the outer and inner layers of the heterocyst envelope, but they will diffuse within a continuous periplasm and are re-imported into the cytoplasm of vegetative cells (Flores et al., 2006). In addition, Nicolaisen et al. (2009) showed that the outer membrane in heterocystous cyanobacteria is an efficient permeability barrier for glutamate and retains this metabolite within the filament. Nevertheless, a lower extracellular pH might hypothetically explain the reduction in N_2 fixation in the Czerny study by restraining the transport of nitrogenous metabolites, but it cannot explain the pronounced decrease in growth rate detected in parallel to a relative small reduction in N_2 fixation rate.

Supporting evidence towards a stimulation of high pCO_2 on growth and production has been published previously for non-heterocystous cyanobacteria of the genus *Trichodesmium* and adjustment of pCO_2 either by aeration with pre-mixed gases (Hutchins et al., 2007; Kranz et al., 2009) or acid/base accomplished with DIC addition (Barcelos e Ramos et al., 2007). In these experiments, growth rates increased from present day (380 ppm) to high pCO_2 (750 ppm to 1000 ppm) scenario by 34 to 38 % and decrease by 30 to 50 % comparing the glacial scenario vs. the present day (Levitan et al., 2007; Barcelos e Ramos et al., 2007). Furthermore, Hutchins et al. (2007) detected no growth in *Trichodesmium* cultures at pCO_2 conditions below 150 ppm.

Stimulation of growth and production rates in our study was lower by an order of magnitude compared to those of *Trichodesmium*. Several researchers observed an elevation of N_2 fixation rates by approximately 35 to 40 % with a maximum of even 400 %, over the respective pCO_2 range (Barcelos e Ramos et al., 2007; Levitan et al., 2007; Hutchins et al., 2007; Kranz et al., 2009). So far, all experiments were conducted in laboratory culture, while field measurements are still scarce. Until today, there is only one publication, Hutchins et al. (2009), which reports a stimulation of cyanobacterial N_2 fixation rates by pCO_2 during three experimental runs (6, 21 and 41 %) in a field population of the Gulf of Mexico.

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The underlying molecular and cell-physiological mechanisms of the beneficial effect of a high pCO_2 environment, however, is still speculative. Levitan et al. (2010a, b) and Kranz et al. (2009, 2010) assume energy savings achieved by down-regulating carbon concentration mechanisms (CCM). The acquisition of carbon in cyanobacteria involves the use of CCM to compensate for a low pCO_2 in aqueous environments, which are typically lower than the half saturation constant of RUBISCO, the major enzyme involved in C fixation. These CCMs often include bicarbonate transporter that allow access to the larger DIC reservoir (Tortell and Morel, 2002). *Trichodesmium*, as well as *Nodularia* both belong to the β-group of cyanobacteria, classified based on the structural differences in RUBISCO (Badger et al., 2002). Both cyanobacteria share CCM components and possess a surplus of one DIC and CO_2 uptake system compared to α-cyanobacteria (BCT1, NADH-I_3). The operation of CCMs is energetically expensive and, because cell membranes are freely permeable for CO_2, additional metabolic costs are incurred in limiting the efflux of CO_2 from the cell. It has been proposed that CCM regulation might occur by changing the gene expression level, but studies by Levitan et al. (2010a, b) and Kranz et al. (2009, 2010) do not support this hypothesis in long-term studies. The discrepancy between CCM gene expression, CCM activity and stimulation of growth and production at high pCO_2 on the other hand, led Levitan et al. (2010b) and Kranz et al. (2009, 2010) suggesting a modulation of the CCM activity at the translational and post-translational level or alteration of the transporter activity. Within this line of arguments, Kranz et al. (2009) showed an increase in activity of a special CCM transporter component at high pCO_2, the NDH-I_4 transporter, a low affinity transporter avoiding efflux of CO_2 from the cytosol by converting CO_2 to HCO_3^-. This elevated activity might lead to enhanced ATP production yielding in an energetic surplus available to fuel N_2 fixation. Regardless of the underlying molecular and cell physiological processes, C and N_2 fixation mechanisms compete for photogenerated reductants and any reduction in energy demand of the C fixation apparatus can be allocated to other metabolic processes including N_2 fixation and would explain the effect of CO_2 availability on potential C, as well as N_2 fixation. A high plasticity of

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CCM regulation in *Trichodesmium* under different pCO_2, but also under variable light and temperature conditions and, moreover, in relation to the current N supply (Giordano et al., 2005) has emerged modulating N and corresponding C demands to keep the respective C:N ratio at a constant level.

Overall, our results suggest a stimulating effect of high pCO_2 on *Nodularia*, which may be broadly applicable to non-heterocystous and heterocystous diazotrophic cyanobacteria of group β unless no other growth factor becomes limiting.

4.2 Nitrogen and carbon turnover and elemental stoichiometry under different pCO_2 conditions

Nitrogen and carbon turnover in our study deviated between the pCO_2 treatments. On the one hand, total nitrogen (TN) within our experimental system increased over 9 days by ~10 µmol l^{-1} in the 180 ppm pCO_2 treatment, by ~20 µmol l^{-1} in the 380 ppm pCO_2 treatment and by ~25µmol l^{-1} in the 780 ppm pCO_2 treatment. On a daily basis, N_2 fixation provided sufficient N to explain the build up of PON and POC. Nevertheless, a discrepancy occurred, because N_2 fixation per day was higher by 0.3 to 1.2 µmol l^{-1} d^{-1} than the build up of PON and thus leading to a surplus of N_{fixed} compared to the build up of PON. DON and DIN exudation was not directly measureable with the sampling interval and method chosen (sampling after 3, 9 and 15 days, exudation calculated from differences in concentration), which might indicate a faster cycling of N compounds than we were able to detect. Moreover, the error of DOM determination of the replicates was in the same concentration range as the differences in concentration from one sampling day to the next. Nevertheless, DON and DIN exudation could explain the discrepancy mentioned above.

There was a constant, although low number of heterotrophic bacteria in our incubation bottle, but our measurements revealed that active growth of bacteria did not occur. Therefore, this bacterial contamination might have resulted from a background of non-viable, but SYBR green stainable bacteria since also extracellular nucleic acids and dead, DNA containing, cells will be stained by the dye. In conclusion, uptake of

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DON and DIN by heterotrophic bacteria should have been negligible. Apart of such potential constrains, we detected a significantly higher mean concentration of DON in the high pCO_2 treatment compared to the glacial scenario (ANCOVA, $p = 0.045$, $n = 12$, 16.6 µmol l^{-1} vs. 15 µmol l^{-1}). Differences between the present day and glacial scenarios, as well as present day and future scenarios were not statistically significant according to ANCOVA, but overall a positive correlation of DON concentration and pCO_2 ($R^2 = 0.622$, $p < 0.01$, $n = 12$) was indicated. This presumes a tendency to elevated exudation of DON at high pCO_2 but it does not give any information on the exact composition of molecules of this DON pool and whether any particular substances are dominating this trend. Some studies have demonstrated that N_2 fixation and subsequent release of nitrogenous organic compounds is a possible mechanism to dissipate excess light energy on a short term scale (Lomas et al., 2000; Wannicke et al., 2009), while no previous report on the effect of pCO_2 on DON release exists.

On the one hand, there are several studies showing that DOC production is insensitive relative to pCO_2 (Engel, 2004; Rochelle-Newall et al., 2004; Grossart et al., 2006). This lack of significant tendencies presumably results from a rapid response of the microbial food web superimposing short term trends of autotrophic processes which might have been significant. On the other hand, Borchard and Engel (2012) recently demonstrated a stimulating effect of greenhouse conditions (high pCO_2 and high temperature) on exudation processes in a laboratory study using *Emiliania huxleyi*. Our model of the daily carbon flow revealed no DOC exudation in the 180 ppm pCO_2 treatment on a daily basis, while there were 6 µmol l^{-1} and 7 µmol l^{-1} DOC released in the 380 ppm and 780 ppm pCO_2 treatments. Furthermore, ratios of newly fixed C:N were above the Redfield ratio and significantly higher than molar C:N ratios of cyanobacteria in the glacial and present day scenarios ($p = 0.03$ and <0.001, respectively, $n = 12$), whereas ratios of newly fixed C:N and molar C:N did not differ in the future scenario ($p = 0.08$, $n = 12$). Moreover, glacial and present scenarios, likewise glacial and future scenarios deviated significantly from each other ($p = 0.02$ and <0.001, respectively, $n = 12$).

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In the high pCO_2 treatment a higher N_2 fixation rate along with a higher C fixation rate suggests synchronic ammonium incorporation into the carbon skeletons (2-oxoglutarat) through the GS-GOGAT (glutamine synthetase-glutamine oxoglutarate aminotransferase) cycle synthesizing glutamate.

POC:POP, as well as PON:POP in this study are elevated relative to the Redfield ratios in all treatments and deviated significantly between 180 ppm vs. 380 ppm and 180 ppm vs. 780 ppm pCO_2 treatments. The positive correlation between POC:POP and PON:POP ratios and biomass (chlorophyll-*a* and abundance) presumes a higher C accumulation relative to N and P and of N relative to P.

In terms of trend and magnitude, our measured elemental ratios are comparable with those given by Hutchins et al. (2007) and Barcelos e Ramos et al. (2007) indicating constant C:N ratios, but an increase in N:P and C:P ratios at high pCO_2. This opposes the trend observed by Levitan et al. (2007), Czerny et al. (2009) and Kranz et al. (2009), who found an increase in C and N quota as well as the ratio at elevated pCO_2.

In general, to date there is no consensus on whether phytoplankton elemental ratios are likely to be altered in a systematic manner in a future acidified ocean. Most of eukaryotic phytoplankton investigated either remained near Redfield values, or increased elemental ratios in a species specific manner (Hutchins et al., 2009; Liu et al., 2010 and references therein). Similarly, natural populations display no clear trend in POM stoichiometry either with increased C:N ratios in some studies (Riebesell et al., 2007; Engel et al., 2005) and a decrease in N:P in others (Tortell et al., 2002; Bellerby et al., 2008).

4.3 Biogeochemical and ecological implications

Seasonally, cyanobacteria in the Baltic Sea exhibit pCO_2 fluctuations with minimum values close to or below the glacial scenario (180 ppm) used in many experiments. In the Gulf of Finland, pCO_2 drops from winter time until May from atmospheric equilibrium values of ~350 ppm to ~150 ppm due to warming of water and increased sequestration by photosynthetic activity (Schneider et al., 2006). This corresponds to a decline

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in pCO_2 of 60 %. In July, pCO_2 concentrations rise slightly up to ~200 ppm and level off again to a minimum of 100 ppm with the onset of the cyanobacteria bloom.

Thus, the natural cyanobacteria community of the Baltic Sea seems to be periodically exposed to glacial like pCO_2 conditions.

If we apply rate measurements obtained in our study in the 180 ppm pCO_2 treatment, growth and production would be lower compared to the present day conditions (380 ppm): by up to 23 % concerning growth rates, by up to 25 % concerning N_2 fixation and by up to 36 % concerning C fixation.

Nevertheless, this C limitation is balanced periodically by upwelling and turbulent mixing of CO_2 and nutrient rich intermediate winter water (Gidhagen, 1987) with CO_2 partial pressures up to 800 ppm (Schneider et al., 2006; Beldowski et al., 2010; Schneider, 2011).

Our results suggest that, as long as growth and production of cyanobacteria in the Baltic Sea are not limited by other factors, e.g. nutrients and light, maximum growth rates of *Nodularia* could potentially rise by 27 % due to the predicted increase in pCO_2 throughout the next 100 yr. The magnitude of C and N_2 fixation rates of *Nodularia*, however, will be lower than hypothesized for *Trichodesmium* until the next 100 yr (Levitan et al., 2007; Barcelos e Ramos et al., 2007) with expected increase of 2 % and 4 %, respectively. Current estimates of N_2 fixation by cyanobacteria are about 125–148 mmol N m^{-2} yr^{-1} for the Baltic Proper (Wasmund et al., 2001a, 2005b). If we assume, that our experimental results can also be extrapolated to the field in the year 2100 this rate could rise to 130–154 mmol N m^{-2} yr^{-1}, caused by the expected increases in pCO_2 alone. Nevertheless, this projected increase remains within the natural variability of rate measurements.

Subsequently, pCO_2 induced increase in N_2 fixation by 4 % would consequently elevate the amount of bioavailable nitrogen by the release of dissolved nitrogenous compounds (DIN and DON) corresponding to a release rate of 104–123 mmol N m^{-2} yr^{-1}, if we apply that 80 % of total nitrogen fixed by cyanobacteria is exudated (Glibert and Bronk, 1994; Ohlendieck, 2000; Wannicke et al., 2009; Ploug et al., 2010). In

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comparison, atmospheric N input to the Baltic Sea accounts for ~80 mmol N m^{-2} yr^{-1} (Larsson et al., 2001), while 45 % of the total N input derives from N_2 fixation. Riverine N load is higher and adds up to 76×10^3 mmol N m^{-2} yr^{-1} (HELCOM, 2005).

Since diazotrophic cyanobacteria can exploit inorganic, as well as organic N sources, they do not solely rely on dissolved inorganic nitrogen sources. Moreover, they still can exploit inorganic phosphorous, as well as organic phosphorous, although dissolved inorganic nitrogen is already limiting. As a result, they drive the ecosystem towards P instead of N limitation. Our results suggest that this phenomenon will be amplified in the future ocean, when rate and extend of mass occurrences of diazotrophs develop, in particular when temperature increases at the same time. Cyanobacteria mass developments not only impact N and P cycling in the phototrophic zone, but also reduce oxygen concentrations in the deeper water layers and on the sediment when their biomass settles out. This will increase oxygen consumption and hence expand hypoxia in the Baltic Sea, which are known to release large quantities of inorganic P (Mort et al., 2010).

In addition to this, we have detected an increase in C:P and N:P ratios at high pCO_2 conditions. Interpolating our results to the Baltic Sea in 2100 suggests that the nutritional value of organic matter produced in the euphotic zone will decrease in the future ocean. This could impact the efficiency of bacterial degradation on the one hand, and zooplankton production on the other hand, affecting the remineralisation potential in deep water layers. Overall, the environmental significance of diazotrophic blooms in the Baltic Sea goes far beyond the detrimental effects of changes in stoichiometry and quantity of degradable biomass to the point of recreational nuisance since changes in toxicity might concur. Eutrophication might play a substantional role in the expansion of cyanobacterial blooms (e.g. O'Neil et al., 2012). The future N input into the Baltic Sea, caused by a pCO_2 induced stimulation of cyanobacteria, might counteract the nitrogen load reductions aimed to mitigate eutrophication (e.g. Vahtera et al., 2007; Voss et al., 2011) and in the worst case impair the socio-economic value of the Baltic Sea.

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Table 1. Carbonate system variables for the four sampling time points. pH and total carbon (C_T) were measured, total alkalinity (A_T) and pCO_2 in seawater were calculated from pH and C_T using CO2SYS (Lewis and Allison, 1998).Values are means and standard deviations of three replicates (except one replicate bottle of the 180 ppm treatment at day 9).

Date	CO_2 treatment [ppm]	Incubation Time [d]	pH NBS scale	C_T [µmol kg^{-1}]	A_T [µmol kg^{-1}]	pCO_2 [µatm]
29 March 2010	180	0	8.02 ± 0.02	1651.9 ± 8.9	1724.2 ± 6.4	472.9 ± 29.9
	380	0	7.95 ± 0.01	1656.8 ± 3.6	1713.4 ± 2.8	561.0 ± 8.7
	780	0	7.93 ± 0.03	1676.7 ± 2.7	1730.3 ± 2.6	590.7 ± 35.3
1 April 2010	180	3	8.16 ± 0.03	1607.4 ± 10.2	1709.7 ± 3.2	330.1 ± 26.3
	380	3	8.09 ± 0.03	1617.5 ± 11.4	1703.5 ± 3.5	388.8 ± 36.0
	780	3	7.94 ± 0.02	1667.9 ± 1.9	1720.5 ± 5.1	576.8 ± 23.3
7 April 2010	180	9	8.22 ± 0.04	1570.4 ± 14.6	1690.8 ± 5.0	276.7 ± 27.1
	380	9	8.18 ± 0.03	1581.1 ± 2.8	1690.9 ± 5.9	305.3 ± 20.3
	780	9	8.07 ± 0.02	1620.7 ± 5.3	1702.3 ± 3.8	412.2 ± 16.9
13 April 2010	180	15	8.20 ± 0.04	1568.0 ± 11.4	1683.3 ± 22.6	291.8 ± 29.3
	380	15	8.14 ± 0.01	1595.9 ± 4.9	1696.7 ± 2.0	339.6 ± 12.5
	780	15	8.03 ± 0.06	1626.9 ± 21.1	1702.2 ± 6.7	452.9 ± 82.2

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Table 2. ANCOVA with time as the covariate and pCO_2 as the nominal predictor (T-, p-value) and Turkey-HSD post hoc analyses (with pCO_2 as predictor). ns: not significant relative to $\alpha = 0.05$. Abbreviations are $N_{2\,fix}$ – N_2 fixation, C_{fix} – C fixation, Chloro.-*a* – chlorophyll-*a*. n = 12 (pCO_2) and 9 (time).

	Abundance	$N_{2\,fix} \cdot l^{-1}$	$C_{fix} \cdot l^{-1}$	Chloro.-*a*	Growth rate	NO_3	PO_4^{3-}	pH	C_T	pCO_2	DOC
Whole model of variance (pO_2 + time)	$F = 8.65$, 0.01	$F = 11.9$, <0.001	$F = 3.97$, 0.01	$F = 8.76$, <0.001	–	$F = 0.346$, 0.89	$F = 1.12$, 0.413	$F = 1,92$, 0.164	$F = 1.05$, 0.44	$F = 3.45$, 0.013	$F = 1.05$, 0.668
pCO_2 (nominal predictor)	$F = 18.94$, <0.001	$F = 4.9$, 0.017	$F = 13.8$ <0.001	$F = 25.7$, <0.001	$F = 31$,	$F = 0.27$, 0.768	$F = 1.07$, 0.375	$F = 49.23$, <0.001	$F = 41.05$, <0.001	$F = 74.6$, <0.001	$F = 0.27$, 0.766
Post Hoc: pCO_2 treatments											
180 vs. 380	ns	ns	< 0.0001	< 0.0001	0.013	ns	ns	ns	ns	0.001	ns
380 vs. 780	0.007	0.045	ns	< 0.0001	¡0.001	ns	ns	< 0.0001	< 0.0001	< 0.0001	ns
180 vs. 780	< 0.0001	ns	0.001	0.006	¡0.001	ns	ns	< 0.0001	< 0.0001	< 0.0001	ns
	DON	DOP	DOC:DON	DOC:DOP	DON:DOP	POC	PON	POP	POC:PON	POC:POP	PON:POP
Whole model of variance (pCO_2 + time)	$F = 0.97$, 0.48	$F = 0.713$, 0.64	$F = 2.08$, 0.141	$F = 0.45$, 0.831	$F = 0.522$, 0.78	$F = 26.33$, <0.001	$F = 21.126$, <0.001	$F = 1.01$, 0.466	$F = 21.09$, <0.001	$F = 22.81$, <0.001	$F = 21.16$, <0.001
pCO_2 (nominal predictor)	$F = 5.18$, 0.026	$F = 0.15$, 0.863	$F = 2.07$, 0.17	$F = 0.262$, 0.77	$F = 0.58$, 0.58	$F = 27.24$, <0.001	$F = 24.288$, <0.001	$F = 1.07$, 0.37	$F = 18.52$, <0.001	$F = 23.99$, <0.001	$F = 24.83$, <0.001
Post Hoc: pCO_2 treatments											
180 vs. 380	ns	ns	ns	ns	ns	< 0.0001	< 0.0001	ns	< 0.0001	< 0.0001	< 0.0001
380 vs. 780	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
180 vs. 780	0.045	ns	ns	ns	ns	< 0.0001	< 0.0001	ns	< 0.0001	< 0.0001	< 0.0001

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Table 3. Abiotic and biotic variables for the four sampling time points. Values are means and standard deviations of three replicates (except one replicate bottle of the 180 ppm treatment at day 9).

Date	CO_2 treatment [ppm]	PO_4^{3-} [$\mu mol\,l^{-1}$]	DIN [$\mu mol\,l^{-1}$]	Chlorophyll-*a* [$\mu g\,l^{-1}$]	Bacterial abundance [$10^5\,l^{-1}$]	DOC [$\mu mol\,l^{-1}$]	DON [$\mu mol\,l^{-1}$]	DOP [$\mu mol\,l^{-1}$]
29 March 2010	180	0.29 ± 0.02	0.22 ± 0.31	0.74 ± 0.08	4.31 ± 2.54	306.5 ± 18.6	16.02 ± 0.07	0.34 ± 0.04
	380	0.32 ± 0.08	0.28 ± 0.16	0.87 ± 0.09	5.61 ± 2.22	295.6 ± 23.9	18.02 ± 1.17	0.33 ± 0.06
	780	0.34 ± 0.02	0.22 ± 0.02	0.71 ± 0.04	3.14 ± 0.22	330.1 ± 76.3	17.67 ± 1.74	0.38 ± 0.06
1 April 2010	180	0.05 ± 0.02	0.41 ± 0.35	3.04 ± 0.12	2.68 ± 0.82	270.6 ± 8.7	15.61 ± 0.01	0.27 ± 0.02
	380	0.03 ± 0.01	0.24 ± 0.03	4.04 ± 0.79	4.95 ± 1.68	297.8 ± 8.5	15.35 ± 0.14	0.24 ± 0.02
	780	0.04 ± 0	0.22 ± 0.02	3.46 ± 0.07	4.33 ± 1.65	296.8 ± 1.6	16.18 ± 1.56	0.28 ± 0.07
7 April 2010	180	0.01 ± 0.01	0.28 ± 0.27	3.38 ± 2.27	5.14 ± 1.56	308.7 ± 19.6	15.44 ± 1.79	0.27 ± 0.08
	380	0.02 ± 0.02	0.20 ± 0.12	5.15 ± 0.23	5.41 ± 2.39	319.9 ± 17.2	15.91 ± 0.82	0.25 ± 0.09
	780	0.01 ± 0	0.14 ± 0.15	7.27 ± 0.42	5.34 ± 3.85	322.6 ± 6.1	15.81 ± 0.44	0.23 ± 0.04
13 April 2010	180	0.03 ± 0.01	0.20 ± 0.04	1.49 ± 0.28	6.62 ± 3.22	318.7 ± 26.8	14.65 ± 0.74	0.24 ± 0.07
	380	0.02 ± 0.01	0.21 ± 0.15	3.05 ± 0.45	2.20 ± 0.33	323.7 ± 16.4	15.13 ± 0.49	0.19 ± 0.02
	780	0.02 ± 0.01	0.10 ± 0	4.41 ± 1.09	6.10 ± 5.24	306.0 ± 15.8	16.79 ± 0.41	0.21 ± 0.04

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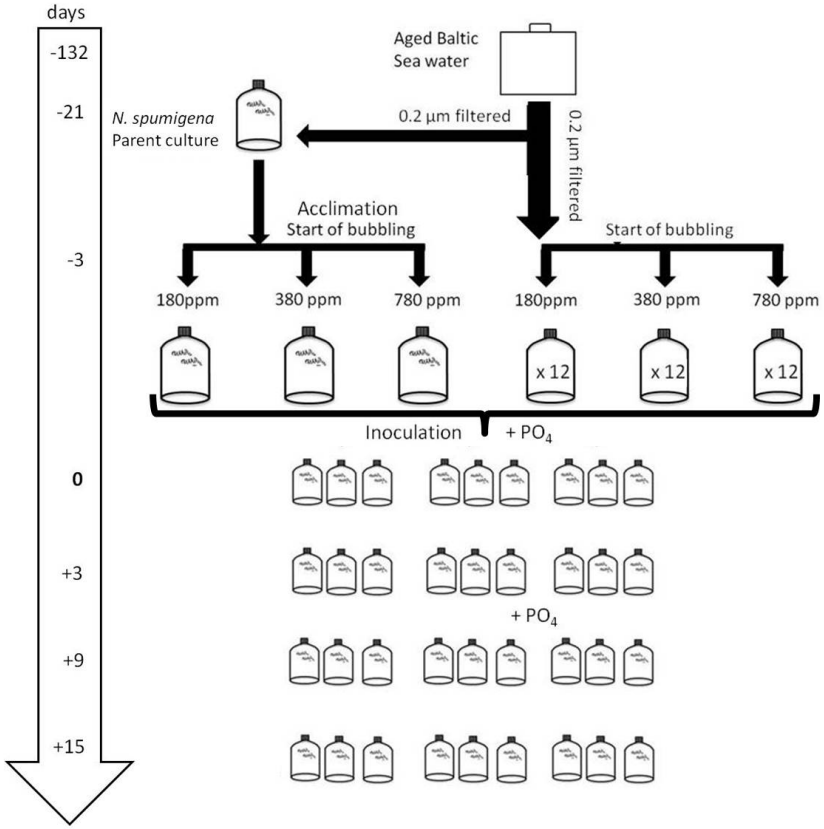

Fig. 1. Schematic overview of the experimental set up and time flow of the single steps taken from preparation (−132 days) to the end of the experiment (+15 days). See text for detailed information.

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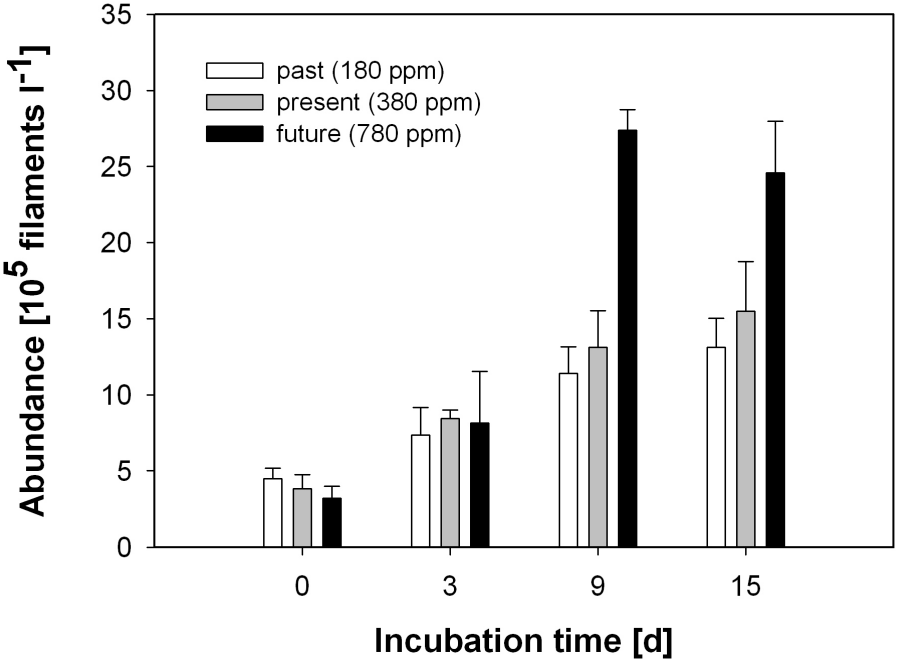

Fig. 2. Changes in abundance with time for the three pCO_2 treatments labelled with the partial pressure of premixed gas with which the cultures were aerated (past pCO_2 = 180 ppm, white bars; present pCO_2 = 380 ppm, grey bars; future pCO_2 = 780 ppm, black bars). Bars represent mean values of three replicates with respective standard deviation.

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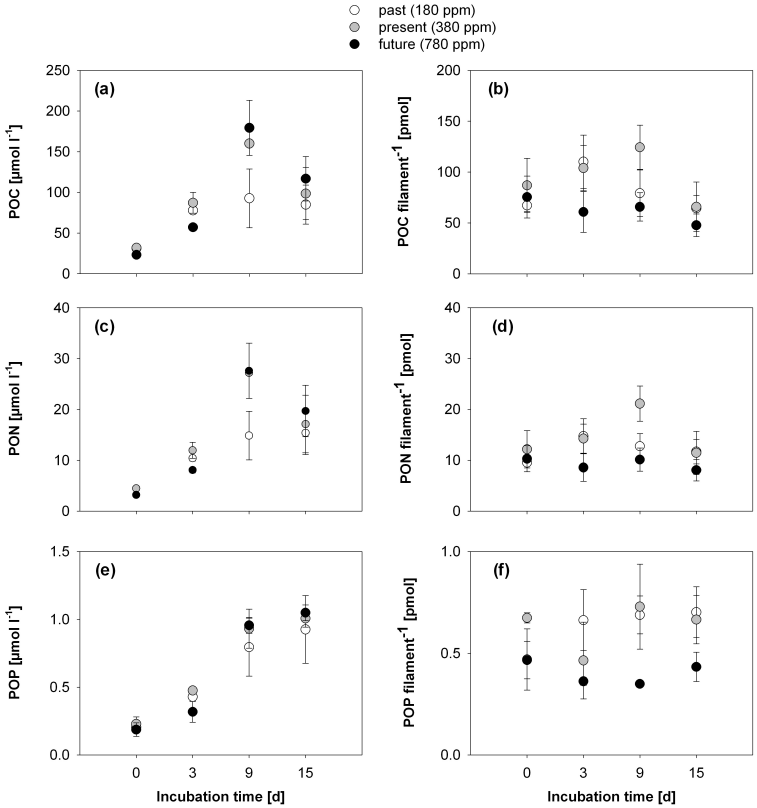

Fig. 3. Time depended variation in particulate organic carbon (POC), particulate organic nitrogen (PON), particulate organic phosphorous (POP) per volume **(a**, **c**, **e)** and per filament (**b**, **d**, **f**) for the three pCO_2 treatments (past pCO_2 = 180 ppm, white circles; present pCO_2 = 380 ppm, grey circles; future pCO_2 = 780 ppm, black circles). Values are means and standard deviations of three replicates.

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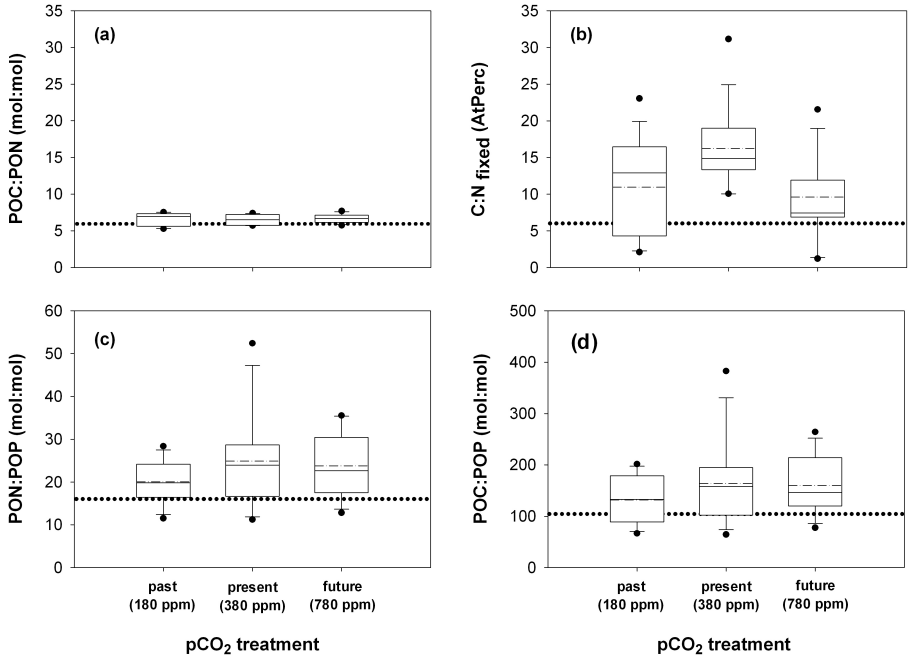

Fig. 4. Box plot ($n = 12$) of particulate organic matter stoichiometry and stoichiometry of carbon to nitrogen fixed for the three pCO_2 treatments (past $pCO_2 = 180$ ppm, present $pCO_2 = 380$ ppm, future $pCO_2 = 780$ ppm). **(a)** Molar ratio of particulate organic carbon to particulate organic nitrogen (POC:PON), **(b)** atom percent ratio of carbon and nitrogen fixed, **(c)** molar ratio of particulate organic carbon to particulate organic phosphorous (POC:POP), **(d)** molar ratio of particulate organic nitrogen to particulate organic phosphorous (PON:POP). Dotted lines represent Redfield stoichiometry. Dashed dotted line represents mean values.

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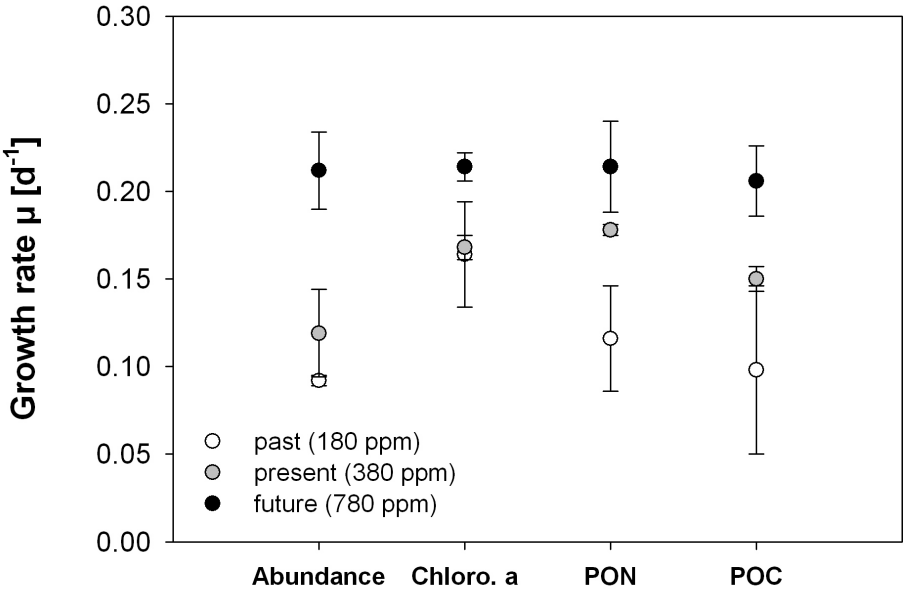

Fig. 5. Calculated growth rates (μ) per day based on changes in abundance, chlorophyll-*a*, particulate organic nitrogen (PON) and particulate organic carbon (POC) for the three pCO_2 treatments (past $pCO_2 = 180$ ppm, triangle; present $pCO_2 = 380$ ppm, square; future $pCO_2 = 780$ ppm, diamond). Symbols represent means of 12 values with standard deviations.

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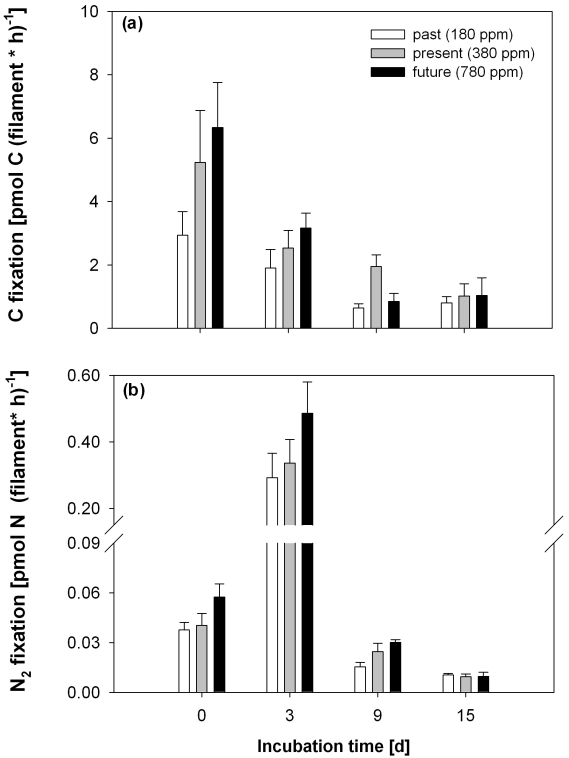

Fig. 6. Changes in filament specific C **(a)** and N_2 fixation rates **(b)** with time for the three pCO_2 treatments (past pCO_2 = 180 ppm, white bars; present pCO_2 = 380 ppm, grey bars; future pCO_2 = 780 ppm, black bars). Bars represent mean values of three replicates with respective standard deviation.

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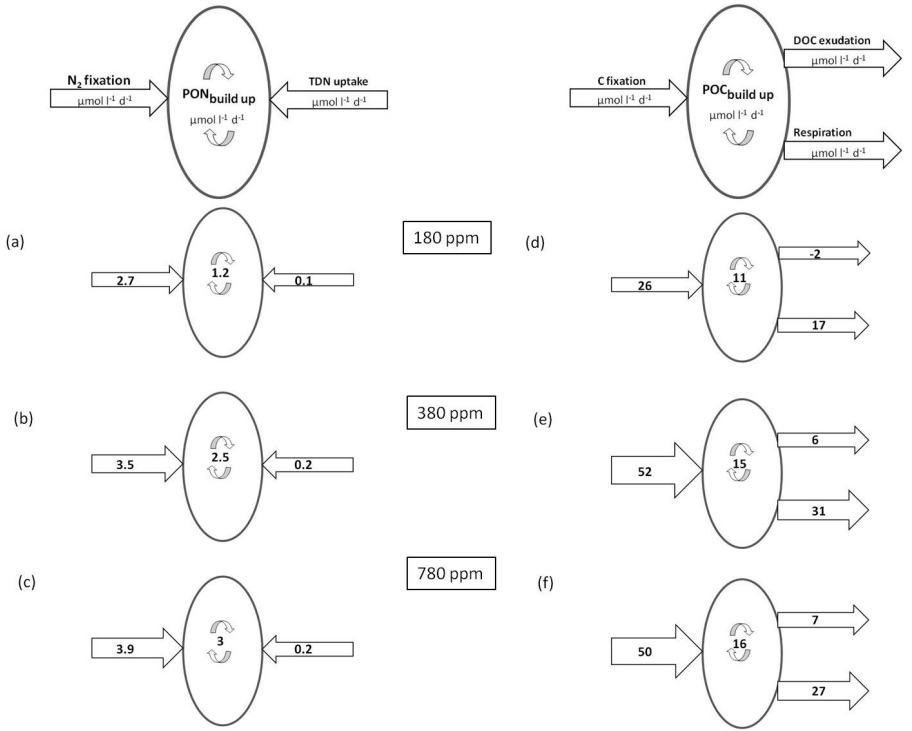

Fig. 7. Basic conceptual model of nitrogen **(a–c)** and carbon **(b–d)** flow within the *Nodularia* culture for the three pCO_2 treatments (past pCO_2 = 180 ppm, present pCO_2 = 380 ppm; future pCO_2 = 780 ppm). Values are means from day 0 to day 9 given in µmol l^{-1} d^{-1}. Respiration in **(b–d)** is calculated from the difference in C fixation and POC build up + DOC exudation. Thicknesses of arrows approximately indicate the magnitude of matter flow.

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