



Baltic Sea diazotrophic cyanobacterium is negatively affected by acidification and warming

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ABSTRACT: Nitrogen fixation is a key source of nitrogen in the Baltic Sea which counteracts nitrogen loss processes in the deep anoxic basins. Laboratory and field studies have indicated that single-strain nitrogen-fixing (diazotrophic) cyanobacteria from the Baltic Sea are sensitive to ocean acidification and warming, 2 drivers of marked future change in the marine environment. Here, we enclosed a natural plankton community in 12 indoor mesocosms (volume ~1400 l) and manipulated partial pressure of carbon dioxide ($p\text{CO}_2$) in seawater to yield 6 CO_2 treatments with 2 different temperature treatments (16.6°C and 22.4°C, $p\text{CO}_2$ range = 360–2030 μatm). We followed the filamentous, heterocystous diazotrophic cyanobacteria community (Nostocales, primarily *Nodularia spumigena*) over 4 wk. Our results indicate that heterocystous diazotrophic cyanobacteria may become less competitive in natural plankton communities under ocean acidification. Elevated CO_2 had a negative impact on *Nodularia* sp. biomass, which was exacerbated by warming. Our results imply that *Nodularia* sp. may contribute less to new nitrogen inputs in the Baltic Sea in the future.

KEY WORDS: Cyanobacteria · Environmental change · Baltic Sea · Nitrogen fixation · *Nodularia spumigena*

INTRODUCTION

The Baltic Sea is a semi-enclosed water body under high anthropogenic stress because of its large and highly populated catchment area (Sweitzer et al. 1996) and long water residence time of over 30 yr due to low water exchange through the Danish Straits (Döös et al. 2004). Eutrophication caused by increased nutrient loads from agricultural run-off has also been of particular concern in the Baltic Sea over the past few decades. This has increased phytoplankton growth, thereby stimulating oxygen consumption and nitrogen (N) loss processes in the deep anoxic basins, and increasing the phosphate pool available for supporting bloom development of filamentous, diazotrophic (N_2 -fixing), and potentially toxic cyanobacteria (Vahtera et al. 2007).

Nodularia spumigena is a filamentous, heterocystous, diazotrophic cyanobacterial species found in

the Baltic Sea which commonly blooms during the N-limited summer period. These extensive surface blooms have been reported as common occurrences since the 1970s (Hübel & Hübel 1974) and form an important annual source of new N. *N. spumigena* and other diazotrophic cyanobacteria assimilate the residual phosphate present after the spring bloom (Kononen et al. 1996) and fix atmospheric N, thereby supplying the wider plankton community with bioavailable N. This N input replenishes N pools which are lost through anammox and denitrification in the anoxic bottom waters (Vahtera et al. 2007) and supports between 20 and 45% of new production in the Baltic Proper annually (Gustafsson et al. 2013).

Climate change introduces new potential drivers of aquatic ecosystem change: anthropogenic carbon dioxide (CO_2) emissions not only warm the atmosphere and the oceans but the CO_2 also dissolves in the surface waters, thereby reducing seawater pH

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(Brewer 1997). These changes are termed ocean warming and ocean acidification, respectively. Increases in summer surface temperature of 2–4°C (von Storch et al. 2015), concurrent with an average surface ocean pH decrease of up to 0.4 pH units (Schneider et al. 2015), are projected for the Baltic Sea by the year 2100. In some regions of the Baltic Sea such as the Kiel Fjord, short-term and seasonal fluctuations already markedly exceed projected changes (Thomsen et al. 2010). These fluctuations may be exacerbated under ocean acidification (Omstedt et al. 2012). Diazotrophic organisms appear to be sensitive to both ocean acidification (Barcelos e Ramos et al. 2007, Fu et al. 2007, Czerny et al. 2009, Wannicke et al. 2012, Hutchins et al. 2013, 2015, Eichner et al. 2014, Gradoville et al. 2014) and warming (Fu et al. 2014) in laboratory studies. However, the response of diazotrophs in field studies with mixed plankton assemblages has been contradictory, with no response to elevated partial pressure of carbon dioxide ($p\text{CO}_2$) in seawater observed in studies in the Pacific Ocean (Law et al. 2012, Böttjer et al. 2014) and the Baltic Sea (A. J. Paul et al. 2016) and a positive response observed in the Mediterranean Sea (Rees et al. 2017).

In this mesocosm study, we wanted to stimulate a bloom of *N. spumigena* and follow (1) the response of diazotroph growth and activity to future ocean acidification and warming scenarios in a natural plankton community and (2) any potential changes in new N inputs. When using a natural community, important factors such as grazing and resource availability and competition will likely modulate bloom initiation and the response of *N. spumigena* to increasing CO_2 , in contrast to the physiological response observed in single-strain culture studies. This experimental set-up used shallow mesocosms (~1.5 m deep) with high light availability, similar to the conditions present in surface waters in the Baltic Sea during summer. There, strong stratification, high light availability and high temperatures facilitate the development of extensive blooms of filamentous diazotrophic cyanobacteria. Based on laboratory experiments using *N. spumigena* (Czerny et al. 2009, Eichner et al. 2014), we expected diazotroph activity and growth to be negatively affected by increased CO_2 under phosphate repletion but positively affected by increased temperature (Suikkanen et al. 2013), a controlling factor of diazotrophy in the Baltic Sea (Wasmund 1997). Furthermore, we anticipated that CO_2 - or temperature-related changes in diazotroph activity and growth will be visible in plankton community biomass due to potential relief of N-limitation in the plankton community.

MATERIALS AND METHODS

Experimental set-up and sampling procedures

This mesocosm study took place using the indoor mesocosm facilities at GEOMAR Helmholtz Centre for Ocean Research Kiel, Germany, between 13 August and 13 September 2013. Four temperature-controlled rooms each held 3 mesocosms with a volume of ~1400 l and surface area of ~1.54 m². We employed an experimental design with 2 temperature treatments (cold = 16.5°C, warm = 22.5°C), each containing 6 target $p\text{CO}_2$ treatments ranging from ambient (~400 μatm) up to 2500 μatm . The temperature in the warm and cold treatments was increased and decreased by 3°C, respectively, from ambient temperature of the Kiel Fjord during mesocosm filling to exclude the effect of any potential temperature shock. The 12 mesocosms were filled simultaneously on 13 August ($t-3 = 3$ d before the onset of the experiment, see also Table S1 in the Supplement at www.int-res.com/articles/suppl/m589p049_supp.pdf) with unfiltered seawater from the Kiel Fjord collected from approximately 2 m water depth. This water contained a natural summer plankton community from the western Baltic Sea including bacteria, phytoplankton and protozoa. To encourage formation of a filamentous diazotrophic cyanobacterial bloom, 1.75 l of *Nodularia spumigena* culture (strain: SCCAP K-1536) was added to each mesocosm on 14 August 2013 ($t-2$). This resulted in an initial cell density of approximately 5100 cells l⁻¹ in each mesocosm. Before addition to the mesocosms, the *Nodularia* sp. culture was cultivated under temperature and light control (18°C, 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) in sterile filtered (0.2 μm) Kiel Fjord water with f/2 nutrients, vitamins and trace elements. Mesozooplankton (90% copepods, mixed species and stages) collected from the Kiel Bight from vertical net catches (10 m depth) were also added to the mesocosms on $t-1$ at a density of 20 ind. l⁻¹, simulating levels reported for this region in the summer season (Behrends 1997) to replenish those lost during mesocosm filling. To acclimate the mesozooplankton to the temperature treatments, collected mesozooplankton were kept in 10 l containers for 24 h in the temperature-controlled rooms. This also enabled dead individuals to be removed before addition to the mesocosms.

A light-permeable polyvinylchloride (PVC) cover on each mesocosm maintained a headspace above the water surface to reduce outgassing of CO_2 during the experiment. An electrical propeller gently mixed the water in each mesocosm and ensured

homogenous distribution of particulate matter and reduced sedimentation. The light intensity and period reflected natural conditions for this latitude and season as calculated by the astronomic model of Brock (1981). The 5 spotlights (HIBay-LED, 100 W each, Lamp unit HL3700 and Profilux II, wavelength range: 400–800 nm) were computer controlled (GHL Groß Hard- und Softwarelösungen). The light:dark cycle of 14.05:9.95 h included a sunrise/sundown simulation of approximately 2 h and an average maximum light intensity of $382.7 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ around noon. Light intensity was measured using a LICOR Li-250A light meter. The experimental time line and a diagram of the mesocosm set-up are provided in Table S1 and Figs. S1 & S2. Regular sampling from all mesocosms took place every Monday, Wednesday and Friday between $t-2$ (Wednesday, 14 August 2013) and $t28$ (Friday, 13 September 2013) between 07:00 and 09:30 h local time. Samples for dissolved inorganic carbon (DIC) were taken directly from the centre of the mesocosms at approximately 0.5 m depth using flexible silicon tubing. Samples for total alkalinity (TA) were collected on the previous evening so that up-to-date TA concentrations could be determined overnight and used on the following sampling day to calculate the required CO_2 enrichments. TA fluctuations over the 48–72 h sampling periods were small and thus the difference in sampling timing to DIC samples had minimal influence on calculation of the CO_2 system. Dissolved organic matter was also sampled directly from the mesocosms using a membrane pump and acid-rinsed tubing to minimise the risk of contamination. Water for all other variables (e.g. particulate matter, dissolved inorganic nutrients) was collected into plastic carboys for subsequent sub-sampling.

Carbonate system manipulations

A range of CO_2 treatments was attained by the addition of CO_2 -saturated seawater. Different amounts of CO_2 -enriched seawater were added to each treatment to set up a range of ~ 420 – $1670 \mu\text{atm}$ average $p\text{CO}_2$ in the warm treatment and ~ 365 – $1920 \mu\text{atm}$ average $p\text{CO}_2$ in the cold treatments (see Table S2 for a summary of total amounts added). CO_2 enrichment started on $t-2$ after sampling and took place every sampling day after general sampling until $t10$. Thereafter, CO_2 -enriched seawater was added every day, apart from $t16$ and $t23$, to maintain more stable CO_2 treatments. Seawater for the CO_2 enrichments

for the whole experiment was collected at the beginning of the study when the mesocosms were being filled, sterile filtered ($0.2 \mu\text{m}$) and stored at 15°C . Water was bubbled with CO_2 gas for at least 6 h on the day of addition. Nutrient concentrations were determined (see 'Analytical procedures' below) on the day of collection as well as on the last day of CO_2 enrichment ($t26$).

Analytical procedures

Carbonate chemistry (TA, DIC)

Samples for TA and DIC concentrations were taken directly from the centre of each mesocosm and gently pressure-filtered (Sarstedt Filtropur PES $0.2 \mu\text{m}$) to exclude particulate material before analysis. DIC samples were collected as gas samples into 50 ml glass flasks (Schott Duran) with at least 100 ml of overflow. TA was analysed by potentiometric titration on an autosampler (Metrohm 869 Sample Changer) and a 907 Titrande Dosing unit according to the open cell method described by Dickson et al. (2007). DIC was analysed by infrared detection of CO_2 by a LICOR LI-7000 on an AIRICA system (MARIANDA). Reported values were calculated as the mean of the 3 best out of 4 measurements with typical precision of $1.5 \mu\text{mol kg}^{-1}$. Certified reference material provided by Andrew Dickson (CRM 115, Scripps Institute for Oceanography of the University of California, San Diego, USA) was used to correct for any drift during analyses for both TA and DIC between sampling days and within a run. Carbonate system parameters and $p\text{CO}_2$ were calculated using measured DIC and TA and the carbonic acid dissociation constants of Millero et al. (2006) in the CO2SYS program (Pierrot et al. 2011), taking into account measured nutrient concentrations, temperature and salinity.

Dissolved inorganic and organic matter

Dissolved inorganic nitrogen ($\text{DIN} = \text{NO}_3^- + \text{NO}_2^-$), phosphate (DIP) and ammonium (NH_4^+) were filtered (cellulose acetate, $0.8 \mu\text{m}$ pore size, Sartorius Stedim) and frozen at -20°C until analysis. Concentrations were determined on an auto-analyser (Skalar, SAN^{PLUS}) as described by Hansen & Koroleff (1999). The actual detection limit varied between sampling days but was on average 0.57 , 0.09 and $0.34 \mu\text{mol l}^{-1}$ for DIN, DIP and NH_4^+ , respectively. Phosphate

excess (P^* , Deutsch et al. 2007) was calculated from DIP, DIN and NH_4^+ concentrations according to:

$$P^* = [\text{DIP}] - ([\text{DIN}] + [\text{NH}_4^+])/16 \quad (1)$$

For dissolved organic nitrogen and phosphorus (DON and DOP, respectively) analyses, 60 ml of samples were filtered through pre-combusted, acid-rinsed GF/F filters (450°C, 6 h) and collected in acid-rinsed, high-density polyethylene (HDPE) bottles and stored at -20°C until analyses. Total dissolved nitrogen and phosphorus were converted to inorganic nitrogen and phosphorus using an autoclave (20 min) and alkaline persulfate oxidation. Concentrations were then determined colorimetrically as described by Hansen & Koroleff (1999). DON concentrations were calculated from total dissolved nitrogen by subtracting DIN and NH_4^+ concentrations. DOP concentrations were calculated from total dissolved phosphate by subtracting DIP concentrations.

Particulate matter (C, N, P)

Particulate matter for C/N and P analyses was collected on GF/F filters (Whatman, nominal pore size of $0.7 \mu\text{m}$, diameter 25 mm) by filtration under reduced vacuum ($<200 \text{ mbar}$) between $t1$ and $t28$. Filtration volumes ranged between 100 and 250 ml to ensure sufficient biomass on the filters for analysis. Samples for analysis by mass spectrometry were stored at -20°C until analysis before they were dried overnight at 60°C and packed into tin capsules. Particulate nitrogen and carbon were converted to N_2 and CO_2 gas, respectively, using the method of Sharp (1974), and the stable isotope ratio ($^{15}\text{N}/^{14}\text{N}$) was analysed on a Finnigan Delta^{plus} isotope ratio mass spectrometer coupled by a ConFlo II to an elemental analyser (EuroEA). In addition to the standard calibration at the beginning of each run, standard materials (caffeine, peptone and acetanilide) were also included within runs to identify any drift and ensure accuracy and full combustion of the samples during analysis. Natural abundance isotope ratios in particulate N ($\delta^{15}\text{N}\text{-PN}$) are reported in per mil (‰) compared to the atmospheric N_2 standard (AIR). Due to analytical problems (SD $>0.2\text{‰}$ in standard material $\delta^{15}\text{N}$) with the samples on $t1$ (biomass too low) and $t28$ (problems with calibration), these data points were excluded from analyses. Total particulate phosphorus concentrations were determined spectrophotometrically as described by Hansen & Koroleff (1999).

Phytoplankton biomass

Phytoplankton abundances for organisms $>5 \mu\text{m}$ were determined to the species level and counted using an inverted light microscope in Lugol-fixed samples (Utermöhl 1958) using 50 ml sedimentation chambers. Common species were counted in diametrical strips using 20×, 40× and 60× lens magnification, depending on cell size. Large and rare species (including *Nodularia*) were counted by scanning the entire bottom plate with a 10× lens. The uppermost volume of the chambers was examined for floating cyanobacterial colonies, which were never found. Phytoplankton biomass ($\mu\text{g C l}^{-1}$) was calculated from abundances using geometric standards (Hillebrand et al. 1999) and carbon content as described by Menden-Deuer & Lessard (2000). Flow cytometry abundances for organisms $<5 \mu\text{m}$ were converted to carbon content using the factors of Sommer et al. (2012). Total phytoplankton biomass was calculated as the combination of phytoplankton carbon content from flow cytometry ($<5 \mu\text{m}$) and microscope ($>5 \mu\text{m}$) estimations. Nostocales biomass is the combined biomass of *N. spumigena* and *Dolichospermum* sp., the 2 most abundant filamentous diazotrophic cyanobacteria observed in this study.

Statistical analyses

We tested the effects of temperature and measured $p\text{CO}_2$ on diazotroph biomass (Nostocales) and indicators of diazotroph activity ($\delta^{15}\text{N}\text{-PN}$, P^* , DOP) by building non-linear mixed-effects (NLME) models using the R package *nlme* (Pinheiro et al. 2015). The experiment was divided into a bloom ($t0\text{--}t10$) and a post-bloom phase ($t12\text{--}t28$). We defined this as the phase when phytoplankton biomass was in decline and below $100 \mu\text{g C l}^{-1}$ on average across all treatments (see also Fig. 3). An NLME model was chosen for this particular analysis because the longitudinal data set included repeated measures, potentially non-constant correlation between observations and non-linear variable response (Lindstrom & Bates 1990).

Nostocales biomass was not normally distributed, so data were log-transformed to satisfy the assumption of normality in the model residuals. We used $p\text{CO}_2$ and temperature as fixed effects to test their influence on diazotroph biomass in the first model ($p\text{CO}_2 \times \text{temperature}$). We then tested the effect of diazotroph biomass, CO_2 and temperature on key biogeochemical indicators ($\delta^{15}\text{N}\text{-PN}$, P^* , DOP) using the model Nostocales biomass $\times p\text{CO}_2 \times \text{temperature}$. In

both models, mesocosm was included as a random effect. We simplified the models to exclude all non-significant terms ($p > 0.05$). Normality of residuals and heteroscedasticity were inspected visually and satisfied model assumptions before performing an ANOVA to test significance of each variable. No collinearity was detected between $p\text{CO}_2$ and Nostocales biomass.

RESULTS

Carbonate system and environmental variables

Temperature treatment levels of $22.4 \pm 0.1^\circ\text{C}$ (warm) and $16.6 \pm 0.4^\circ\text{C}$ (cold) were reached (mean \pm SD) by t_2 and remained within 0.4°C of these values until the end of the experiment on t_{28} . A gradient of CO_2 treatments was present from t_3 onwards, with average $p\text{CO}_2$ ranging from 420 to 1760 μatm in the warm treatment, and from 360 to 2030 μatm in the cold treatment (Fig. 1A,B). However, there was a high level of variability within each treatment, particularly in the highest $p\text{CO}_2$ treatments primarily due to the high concentration difference to the atmospheric level and corresponding high level of out-gassing in the shallow mesocosms. Daily additions of CO_2 -enriched seawater after t_{10} (except for t_{16} and t_{23}) improved stability within each treatment.

Salinity ranged between 15.2 and 15.3, and TA remained relatively stable and ranged between 1950 and 1970 $\mu\text{mol kg}^{-1}$. There was a small TA drawdown later in the study period during the post-bloom phase (Fig. 1C,D). This appeared to be slightly higher at lower CO_2 and higher temperature.

Filamentous diazotrophic cyanobacterial biomass and contribution to phytoplankton community biomass

Nodularia spumigena was the dominant filamentous diazotrophic cyanobacterium identified in all mesocosms. Biomass increased with additions of *N. spumigena* culture to all mesocosms with an average biomass on t_0 of 0.62 ± 0.09 and $0.62 \pm 0.11 \mu\text{g C l}^{-1}$ (mean \pm SD) in the warm and cold treatments, respectively (Fig. 2A,B). The next dominant species identified in the order Nostocales was *Dolichospermum* sp., which contributed on average to less than 15% of Nostocales biomass ($N. spumigena = 0.32 \pm 0.32 \mu\text{g C l}^{-1}$, *Dolichospermum* = $0.05 \pm 0.10 \mu\text{g C l}^{-1}$, mean \pm SD), but up to 100% of biomass in some

warm treatments during the post-bloom phase when *N. spumigena* was no longer present.

Total phytoplankton biomass declined during the experiment and remained low ($89.7 \pm 66.7 \mu\text{g C l}^{-1}$, mean \pm SD; Fig. 3A,B) and within the range of reported values for the Kiel Fjord (Wasmund et al. 2008). There was a higher variability in starting phytoplankton community biomass on t_{-2} between mesocosms in the cold treatment ($157.6 \pm 49.5 \mu\text{g C l}^{-1}$) than in the warm treatment ($168.3 \pm 13.1 \mu\text{g C l}^{-1}$). Nostocales biomass declined over time, and the maximum biomass ($<1.5 \mu\text{g C l}^{-1}$) remained well below the definition of a bloom of $22 \mu\text{g C l}^{-1}$ according to Wasmund (1997). The observed biomass was also considerably lower than other studies with artificial blooms ($\sim 460 \mu\text{g C l}^{-1}$, Engström-Öst et al. 2002) and contributed on average to less than 3% of total phytoplankton biomass throughout the study period. In some cold treatment mesocosms, the contribution of Nostocales to phytoplankton community biomass increased after t_{20} to up to 23% (Fig. 2C,D).

No direct effect of temperature was detected, but there was a significant effect of CO_2 on Nostocales biomass in both the bloom and post-bloom periods and in both temperature treatments. A highly significant interactive effect between CO_2 and temperature was detected ($p < 0.0001$, Table 1). Nostocales biomass was generally highest under low CO_2 and low temperature and lowest under high CO_2 and high temperature (Fig. 2). An increase in Nostocales biomass after the *N. spumigena* culture addition on t_0 was only discernible in the lowest CO_2 treatments.

Indicators of diazotrophic activity

While N_2 -fixation rates were analysed following a method with minor modifications from Mohr et al. (2010), ^{15}N - N_2 gas contaminated with ^{15}N -labelled ammonium, nitrate and/or nitrite was used (Dabundo et al. 2014) and thus the measured rates are considered unreliable for this study. Nevertheless, we analysed $\delta^{15}\text{N}$ -PN, DOP and P^* as indicators of diazotrophic activity as described in the following 2 sections.

Stable isotope ratios in particulate nitrogen

$\delta^{15}\text{N}$ -PN decreased from $3.5 \pm 1.2\text{‰}$ (mean \pm SD) on t_3 to reach a minimum of $-0.2 \pm 1.2\text{‰}$ on t_{10} before increasing again in the post-bloom period to reach $2.2 \pm 1.7\text{‰}$ on t_{26} across all CO_2 and temperature treatments (Fig. 2E,F). There was a significant

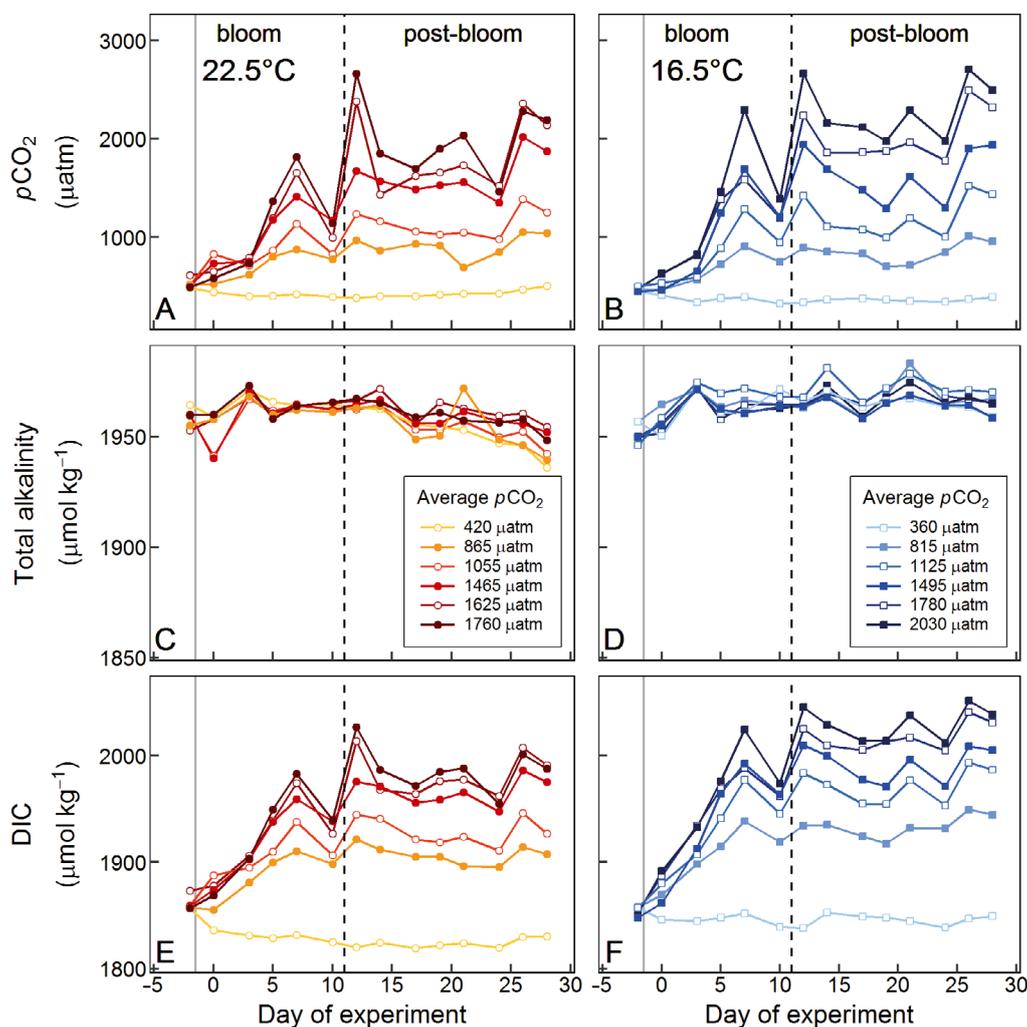


Fig. 1. Carbonate system variables during the study period. Calculated $p\text{CO}_2$, measured total alkalinity and dissolved inorganic carbon (DIC). Grey vertical lines indicate when the *Nodularia spumigena* culture was added to the mesocosms, and the black dashed vertical lines indicate the division between bloom and post-bloom phases

negative effect of temperature on $\delta^{15}\text{N}$ -PN in both the bloom and post-bloom periods ($p = 0.0054$ and $p = 0.0008$, respectively; Table 2), but no significant effect of CO_2 . In the post-bloom period, there was a significant interaction between CO_2 and Nostocales biomass on $\delta^{15}\text{N}$ -PN ($p < 0.0001$, Table 2), even though there was no significant effect of either factor independently.

Inorganic and organic nutrient concentrations

Nutrient concentrations directly after mesocosms were filled on $t-3$ were 0.60 , 1.83 and $0.66 \mu\text{mol l}^{-1}$ for DIN, NH_4^+ and DIP, respectively. DIN and NH_4^+ concentrations in almost all samples were below the

detection limits, which were on average 0.60 and $0.34 \mu\text{mol l}^{-1}$, respectively, as is common for the summer season in the Kiel Fjord (Smetacek 1985). There was an excess of inorganic phosphate (P^*) in all mesocosms throughout the study period (Fig. 3C,D; $0.33 \pm 0.10 \mu\text{mol l}^{-1}$, mean \pm SD), compared to inorganic N according to the Redfield ratio (Redfield 1958). During the bloom phase, Nostocales biomass, $p\text{CO}_2$ and temperature all had a significant negative effect on P^* (Table 2), whereas in the post-bloom period, only temperature still had a significant negative effect ($p < 0.0001$). In addition, there was a positive interactive effect of Nostocales biomass and temperature on P^* during the post-bloom phase.

Through the regular additions of CO_2 -enriched seawater to maintain more constant $p\text{CO}_2$ in the

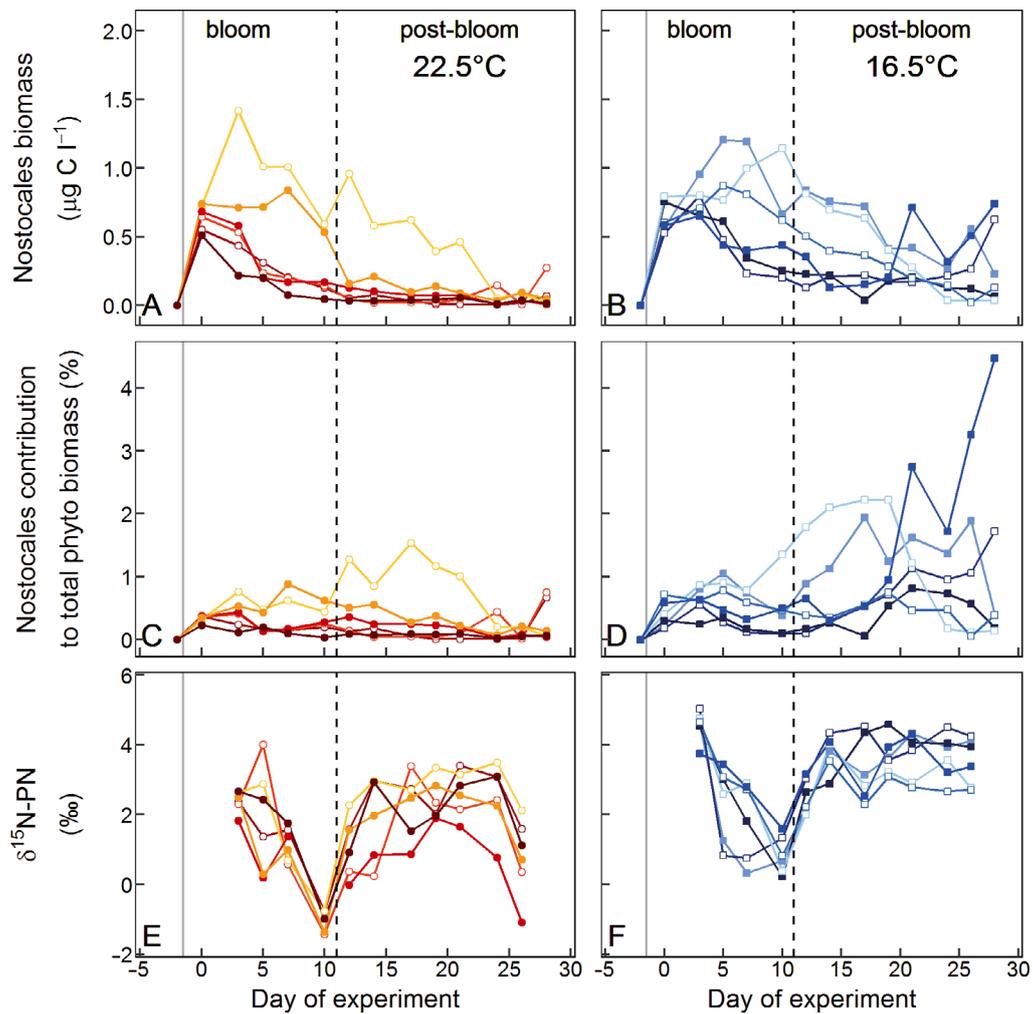


Fig. 2. (A,B) Development of filamentous diazotrophic cyanobacterial biomass (order Nostocales), calculated from microscopy counts of *Nodularia spumigena* and *Dolichospermum* sp. using reported biovolumes (Olenina et al. 2006) and cellular carbon content (Menden-Deuer & Lessard 2000), and (C,D) % contribution to phytoplankton community biomass. (E,F) $\delta^{15}\text{N-PN}$ (‰) is reported as potential indicator of diazotroph activity during the study period. Colours, symbols and lines are as described in Fig. 1

mesocosms, we also inadvertently added small amounts of inorganic nutrients (NH_4^+ , DIN and DIP). However, total amounts added during the study period were small ($0.02 \mu\text{mol l}^{-1}$ DIN, $0.02 \mu\text{mol l}^{-1}$ DIP, $0.07 \mu\text{mol l}^{-1}$ NH_4^+) and remained well below the ambient N pool size (e.g. DON $\sim 17 \mu\text{mol l}^{-1}$, PON $\sim 2 \mu\text{mol l}^{-1}$) in the mesocosms. We consider the minor nutrient input during CO_2 manipulations to have had a negligible effect on Nostocales biomass with negligible relief of N-limitation of the phytoplankton present. Amounts of added NH_4^+ were not of a magnitude shown to affect *N. spumigena* growth or activity (Huber 1986, Sanz-Alferez & del Campo 1994, Lehtimaki et al. 1997, Vintila & El-Shehawy 2010).

DOP concentrations in the mesocosms were on average $0.32 \pm 0.08 \mu\text{mol l}^{-1}$ (mean \pm SD) over the

entire study period, and there was no significant effect of Nostocales biomass, $p\text{CO}_2$ or temperature (data not shown).

DISCUSSION

Influence of elevated seawater CO_2 and temperature on diazotroph abundances

Rising seawater CO_2 led to lower biomass of filamentous diazotrophic cyanobacteria, predominantly *Nodularia spumigena* and *Dolichospermum* sp., with excess phosphate present. Thus, this study using a natural Baltic Sea plankton community agrees with the results of 2 monoculture studies which reported

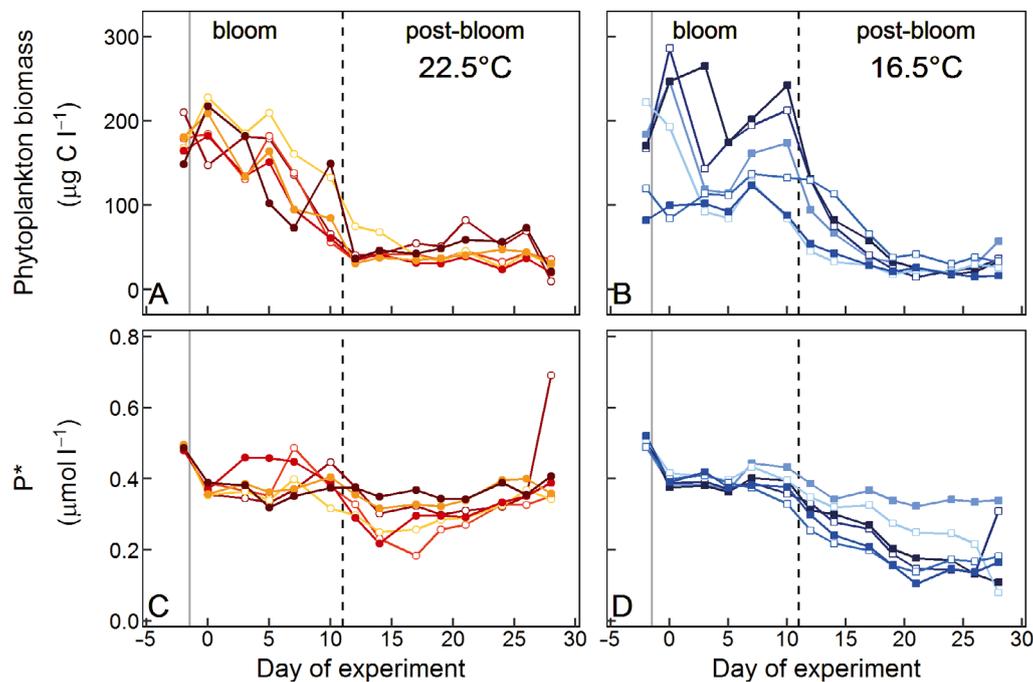


Fig. 3. Phytoplankton community biomass, calculated from microscopy of phytoplankton abundances using reported biovolumes (Olenina et al. 2006) and cellular carbon content (Menden-Deuer & Lessard 2000), and excess dissolved inorganic phosphate (P^*) dynamics during the study period. Colours, symbols and lines are as described in Fig. 1

Table 1. Summary of detected significant fixed effects in mixed effects model analyses for diazotroph biomass (Nostocales). The initial model tested ($pCO_2 \times$ temperature) was simplified to remove all insignificant fixed effects. Bloom and post-bloom indicate $t-2$ (i.e. 2 d before onset of the experiment) to $t10$ (10 d after), and $t12$ to $t28$, respectively. Nostocales was log-transformed to satisfy the assumption of normally distributed residuals in the simplified model. Values indicate F -statistics. Arrows indicate direction of the response, with ** $p < 0.01$ and *** $p > 0.001$

Significant fixed effect	log(Nostocales biomass)	
	Bloom (df = 46)	Post-bloom (df = 82)
pCO_2	39.24 *** ↓	9.56 ** ↓
$pCO_2 \times$ temperature	23.52 *** ↓	57.04 *** ↓

lower growth rates in *N. spumigena* with rising CO_2 (Czerny et al. 2009, Eichner et al. 2014). The small artificial *N. spumigena* bloom was sustained for longer in the lowest CO_2 treatments in both temperature treatments. An increase in observed biomass indicates net growth in these treatments during the bloom phase (Fig. 2A,B). In contrast, there was no clear period of biomass accumulation or bloom development in any CO_2 treatment above 1000 μatm (average treatment pCO_2) in the warm treatment.

The CO_2 treatment levels selected here of up to an average of $\sim 2000 \mu atm$ spanned a much wider range than the widely recognised projections by 2100 of up to 1000 μatm (Collins et al. 2013) but are within the natural range of the Kiel Fjord which can reach up to 3000 μatm during late summer (Thomsen et al. 2010). Nevertheless, the strongest biomass response to pCO_2 appeared to be between ambient and approximately 1000 μatm . This indicates a potential pCO_2 threshold for net Nostocales growth. Similarly, the most marked changes in diazotroph activity and growth in a variety of oceanic taxa were evident within a comparable, and ocean acidification-relevant, pCO_2 range (Hutchins et al. 2013). Based on both our study and results from Hutchins et al. (2013), we suggest that the most critical changes in diazotroph physiology may occur within the range of realistic future average pCO_2 levels (under 1000 μatm).

Rising temperature has been proposed as a key driver of the observed increased filamentous cyanobacterial presence in summer in the Baltic Sea (Suikkanen et al. 2013), with suggestions that future warming will continue this positive trend (HELCOM 2013). Warmer temperatures promote growth in slow-growing diazotrophs, increasing their competitiveness with other autotrophic organisms in plank-

Table 2. Summary of detected significant fixed effects in mixed effects model analyses of and indirect indicators of diazotroph activity ($\delta^{15}\text{N}$ in particulate nitrogen [$\delta^{15}\text{N-PN}$] and excess dissolved inorganic phosphate concentration [P^*]). The initial model tested (Nostocales biomass \times $p\text{CO}_2$ \times temperature) was simplified to remove all insignificant fixed effects. Bloom and post-bloom indicate $t-2$ to $t10$ and $t12$ to $t28$, respectively. Values indicate F -statistics, and dashes (–) indicate that the fixed effect had no significant effect on the variable. Arrows indicate direction of the response, with ** $p < 0.01$, *** $p > 0.001$

Significant fixed effect	$\delta^{15}\text{N-PN}$		$[\text{P}^*]$	
	Bloom (df = 35)	Post-bloom (df = 69)	Bloom (df = 55)	Post-bloom (df = 81)
Nostocales biomass		–	23.70 *** ↓	–
$p\text{CO}_2$		–	20.89 *** ↓	
Temperature	8.81 ** ↓	12.24 *** ↓	8.79 ** ↓	19.61 *** ↓
Nostocales biomass \times $p\text{CO}_2$		16.50 *** ↓		
Nostocales biomass \times temperature				7.30 ** ↑

ton communities (Paerl 2012) and thereby stimulate blooms of *N. spumigena* in the field (Kononen et al. 1996, Pliński & Józwiak 1999). However, we could not identify any direct relationship between temperature and Nostocales biomass, in either the bloom or post-bloom phases (Table 1), even though the warm temperature treatment (22.4°C) was within the reported optimum temperature range of 20–25°C for *N. spumigena* growth (Lehtimäki et al. 1994). The only significant temperature effect observed was an interactive effect between temperature and CO_2 concentrations: the negative effect of increasing CO_2 on Nostocales biomass was exacerbated at higher temperatures in both the bloom and post-bloom phases.

Temperature is generally considered to have a more marked impact on plankton growth than seawater $p\text{CO}_2$ based on studies on plankton community responses (Hare et al. 2007, Coello-Camba et al. 2014, Maugendre et al. 2015, Sommer et al. 2015), growth parameters in single strain culture experiments in the laboratory (Fu et al. 2007) and using structural equation modelling (Alsterberg et al. 2013). Indeed, the response of Nostocales to elevated CO_2 but not elevated temperature that we observed here is contrary to the response of the wider plankton community in this experiment (C. Paul et al. 2016). This may be due, at least in part, to the experimental design used in this study. Unlike previous mesocosm experiments, where $p\text{CO}_2$ has fluctuated with the plankton bloom dynamics (Engel et al. 2008, Schulz et al. 2013), almost daily additions of CO_2 -enriched seawater after $t10$ were used to maintain $p\text{CO}_2$ levels as close to target values as possible. Thus, the mainte-

nance of CO_2 treatments, and hence maintenance of treatment stress, may have contributed to the comparatively strong CO_2 response in diazotroph biomass in this study. However, we would be careful to extrapolate this lack of temperature effect on Nostocales growth that we observed outside the framework of this specific experimental set-up. In particular, there is substantial evidence and widespread agreement in the literature on the critical role of temperature on growth of filamentous diazotrophic cyanobacteria (Staal et al. 2003, Breitbarth et al. 2007, Paerl & Huisman 2008), including in the Baltic Sea (Wasmund 1997, Suikkanen et al. 2013).

Negative effect of ocean acidification on diazotroph biomass not reflected in diazotroph activity

During the period of highest Nostocales biomass ($t3-t10$), $\delta^{15}\text{N-PN}$ decreased in all treatments. This may indicate increased uptake of isotopically light N, potentially through fixation of low $\delta^{15}\text{N}$ from atmospheric N_2 by diazotrophs, into plankton community biomass. Yet there is no direct evidence that $\delta^{15}\text{N-PN}$, a proxy for diazotroph activity, was directly affected by CO_2 , despite the strong negative response to CO_2 observed in Nostocales biomass (Tables 1 & 2; see also discussion in previous section).

Temperature had a more significant influence on $\delta^{15}\text{N-PN}$ than CO_2 , with warmer temperature having a negative effect on $\delta^{15}\text{N-PN}$. This temperature effect on this potential diazotroph activity proxy could not be attributed to higher diazotroph biomass in the higher temperature treatment (Tables 1 & 2). Additional factors such as a high importance of regenerative production (Sigman et al. 2009), differential nitrate isotope fractionation between phytoplankton species (Needoba et al. 2003) and uncertainty in inorganic N concentration, also complicate interpretation of the potential diazotrophic $\delta^{15}\text{N}$ signature in $\delta^{15}\text{N-PN}$. In this study, there were higher zooplankton abundances (C. Paul et al. 2016), and presumably also higher zooplankton organic matter respiration, in the warmer treatments. Inorganic N concentrations were below the detection limit, and therefore their influence on $\delta^{15}\text{N-PN}$ was assumed to be negligible. Hence, increased regeneration of

isotopically light inorganic nitrogen by zooplankton may better explain variation in $\delta^{15}\text{N}$ -PN than diazotroph activity.

The response of other indicators of diazotroph activity, such as P^* , to CO_2 and temperature are also contradictory. For example, Nostocales biomass had a significant negative correlation with P^* during the bloom phase (Table 2), fitting with the idea from Deutsch et al. (2007) that diazotrophs add N, use P and thereby reduce P^* . However, P^* was also negatively correlated with both CO_2 and temperature independently during the bloom phase (Table 2). This would suggest higher N_2 -fixation under higher CO_2 and higher temperature, thus contradicting the negative relationship detected between Nostocales biomass and CO_2 and $\text{CO}_2 \times$ temperature (Table 1). These opposing responses indicate that Nostocales were not shaping the response of P^* to CO_2 and temperature. Reasons for this could be their low contribution of diazotrophic cyanobacteria to plankton biomass, or the nutrient uptake of other more abundant, non-diazotrophic plankton. Thus, while diazotroph biomass was significantly affected by elevated CO_2 , we have no clear indication of any temperature or CO_2 effect on diazotroph activity within this 28 d long study period.

Potential consequences for future diazotrophic N supply to the Baltic Sea

Despite warm temperatures ($>16^\circ\text{C}$), light and phosphate availability fitting the suggested requirements for diazotrophic growth in the Baltic Sea (Wasmund 1997), no large bloom of Nostocales developed in our study. Nostocales generally contributed to less than 1% of total phytoplankton biomass (Fig. 2C,D). This is considerably less than in field studies reporting large surface aggregates contributing around 20 to 30% of plankton community biomass (Stal et al. 1999, Andersson et al. 2015); consequently, their influence on biogeochemical pools was limited. The lack of bloom development could also be due to experimental artefacts. In order to control temperature, this study took place indoors, where we mimicked natural light conditions using an artificial light source with no UV radiation. Lack of UV radiation can influence phytoplankton community composition (Mousseau et al. 2000) and cell stoichiometry (Hessen et al. 2008), and can modulate the physiological response of phytoplankton to ocean acidification (Gao et al. 2009). Additionally, the well-mixed mesocosms may have provided less favourable conditions

for *N. spumigena*, which usually bloom in highly stratified water columns (Wasmund 1997).

The negative growth response of the dominant diazotrophic cyanobacterium in this study, *N. spumigena*, to increasing CO_2 reported in some physiological studies was verified here in a natural plankton community. The direction of the negative physiological CO_2 response in this Baltic Sea cyanobacterium was not modified through resource competition with other phytoplankton functional groups or by top-down grazing pressure. If this negative CO_2 effect on biomass cascades through the food web over longer time periods, it may lead to a decrease in diazotroph N inputs, with consequences for productivity in the wider plankton community.

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