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Diversity of ocean acidification effects on marine N_2 fixers

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ABSTRACT

Considering the important role of N_2 fixation for primary productivity and CO_2 sequestration, it is crucial to assess the response of diazotrophs to ocean acidification. Previous studies on the genus *Trichodesmium* suggested a strong sensitivity towards ocean acidification. In view of the large functional diversity in N_2 fixers, the objective of this study was to improve our knowledge of the CO_2 responses of other diazotrophs. To this end, the single-celled *Cyanothece sp.* and two heterocystous species, *Nodularia spumigena* and the symbiotic *Calothrix rhizosoleniae*, were acclimated to two pCO_2 levels (380 vs. 980 µatm). Growth rates, cellular composition (carbon, nitrogen and chlorophyll *a*) as well as carbon and N_2 fixation rates (^{14}C incorporation, acetylene reduction) were measured and compared to literature data on different N_2 fixers. The three species investigated in this study responded differently to elevated pCO_2, showing enhanced, decreased as well as unaltered growth and production rates. For instance, *Cyanothece* increased production rates with pCO_2, which is in line with the general view that N_2 fixers benefit from ocean acidification. Due to lowered growth and production of *Nodularia*, nitrogen input to the Baltic Sea might decrease in the future. In *Calothrix*, no significant changes in growth or production could be observed, even though N_2 fixation was stimulated under elevated pCO_2. Reviewing literature data confirmed a large variability in CO_2 sensitivity across diazotrophs. The contrasting response patterns in our and previous studies were discussed with regard to the carbonate chemistry in the respective natural habitats, the mode of N_2 fixation as well as differences in cellular energy limitation between the species. The group-specific CO_2 sensitivities will impact differently on future biogeochemical cycles of open-ocean environments and systems like the Baltic Sea and should therefore be considered in models estimating climate feedback effects.

1. Introduction

Owing to their ability to fix atmospheric N_2, diazotrophic cyanobacteria play a crucial role in the biogeochemical cycles of nitrogen as well as carbon. N being the limiting nutrient in vast regions of the ocean (e.g. Moore et al., 2013), N_2 fixers fuel primary productivity by providing a source of new N to the phytoplankton community. While primary production based on remineralization in the upper mixed layer does not lead to C export, at least on longer time and spatial scales, the so-called new production based on N_2 fixation can significantly foster net CO_2 sequestration (Eppley and Peterson, 1979). Since nutrient concentrations in the low latitude surface ocean are expected to decrease with the predicted increase in stratification, N_2 fixation may become more important as global warming progresses (Doney, 2006). Aside from global warming, rising atmospheric CO_2 levels cause ocean acidification, which can affect phytoplankton in numerous ways (e.g. Rost et al., 2008). Considering the important role of N_2 fixation for primary productivity and CO_2 sequestration, it is crucial to assess the response of diazotrophs to ocean acidification. Laboratory experiments on the abundant diazotroph *Trichodesmium sp.* suggested a strong CO_2 sensitivity for this species, with an increase in N_2 fixation or particulate organic nitrogen (PON) production ranging between 35 and 140% for pCO_2 levels expected by the end of this century (e.g. Hutchins et al., 2007; Kranz et al., 2011; Levitan et al., 2007). While *Trichodesmium* is undoubtedly the most prominent diazotroph in the current ocean, contributing up to 50% of marine N_2 fixation (Mahaffey et al., 2005), the development of new methods has shed light on the importance of previously unrecognized N_2 fixers (e.g. Moisander et al., 2010; Thompson and Zehr, 2013; Zehr, 2011).

Diazotrophs differ with regard to cellular structure and physiological key mechanisms, which are linked to the different strategies they evolved to protect their N_2 fixing enzyme, nitrogenase, from O_2. Single-celled species generally separate photosynthesis and N_2 fixation in time, fixing N_2 only during the night, whereas photosynthesis is carried out during the day. This day-night-cycle requires a concerted

Abbreviations: CCM, carbon concentrating mechanism; chl *a*, chlorophyll *a*; DDA, diatom-diazotroph-association; DIC, dissolved inorganic carbon; POC, particulate organic carbon; PON, particulate organic nitrogen; TA, total alkalinity.

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regulation of nitrogenase synthesis and degradation as well as respiration and photosynthesis, which is driven largely by a circadian rhythm (Mohr et al., 2010; Sherman et al., 1998; Toepel et al., 2009). Photosynthetic products are stored within glycogen granules that are broken down by respiration during the night, providing ATP for the highly energy-demanding process of N_2 fixation (Saito et al., 2011; Schneegurt et al., 1994). In most filamentous species, photosynthesis and N_2 fixation are separated in space, with only certain cells within a filament containing nitrogenase. These heterocysts are fully differentiated cells that lack photosystem II and are surrounded by a thick cell wall, which allows for N_2 fixation during the day by posing a diffusion barrier to O_2. N fixed in these cells is transported along the filament in the form of amino acids, while heterocysts are supplied with carbohydrates by the vegetative cells (reviewed in Böhme, 1998; Kumar et al., 2010). In *Trichodesmium*, photosynthesis and N_2 fixation are separated in space as well as time, with so-called diazocytes fixing N_2 during midday when photosynthesis is typically down-regulated (Berman-Frank et al., 2001; Fredriksson and Bergman, 1997).

The role of unicellular diazotrophs may have been severely underestimated, in terms of their ecology as well as biogeochemistry. Field studies using molecular tools to target nitrogenase genes have revealed high abundances of small, unicellular diazotrophic cyanobacteria (UCYN), which may contribute a significant share of marine N_2 fixation (Langlois et al., 2005; Moisander et al., 2010; Montoya et al., 2004; Zehr et al., 1998). Cyanobacteria belonging to group UCYN-A, which lack the genes for photosystem II and RubisCO and are therefore assumed to be symbiotic (Zehr et al., 2008), appear to be highly abundant, their global *nifH* gene abundance exceeding that of *Trichodesmium* (Luo et al., 2012). While most of these newly discovered species are uncultivated and thus poorly characterized, the physiology of *Crocosphaera* (UCYN-B) and *Cyanothece* (closely related to group UCYN-C) has been investigated in laboratory experiments. Heterocystous diazotrophs play an important ecological role in many fresh water as well as brackish systems, not only due to their ability to fix N_2 but also due to their tendency to form extensive, partly toxic blooms. In the Baltic Sea, for instance, blooms formed by *Nodularia spumigena, Aphanizomenon sp.* and *Anabaena sp.* have attracted attention for many decades primarily due to their broad impact on the ecosystem and nuisance to humans (Sivonen et al., 1989, 1990; Stal et al., 2003). The dense surface scums building up during blooms form highly productive microenvironments (Ploug, 2008) and provide a significant source of N to the ecosystem (Larsson et al., 2001). In open ocean environments, heterocystous species have been observed to form symbioses with diatoms. These so-called diatom-diazotroph-associations (DDAs) reach especially high abundances in the tropical Atlantic Ocean, where they benefit from nutrient inputs by the Amazon River plume (Carpenter et al., 1999; Foster et al., 2007; Luo et al., 2012; Subramaniam et al., 2008; Villareal, 1994). Global N_2 fixation rates by DDAs have been tentatively estimated to equal those of *Trichodesmium* (Foster et al., 2011). These symbioses may furthermore be especially important in the context of the biological C pump, as the heavy silica shells of the diatoms act as ballast material (Karl et al., 2012; Subramaniam et al., 2008; Yeung et al., 2012). Symbiotic heterocystous cyanobacteria identified to date include *Richelia intracellularis* found in symbioses with diatoms of the genera *Rhizosolenia* and *Hemiaulus*, and *Calothrix rhizosoleniae,* a symbiont of *Chaetoceros*, which has been isolated and successfully maintained in lab cultures (Foster et al., 2010). While relatively little is known about the physiological interactions between symbiont and host, cyanobacteria in symbioses have been shown to fix N_2 in excess of their needs and transport large amounts of fixed N to the host diatoms (Foster et al., 2011).

While a number of studies addressed the CO_2 sensitivity of *Trichodesmium* (e.g. Barcelos é Ramos et al., 2007; Hutchins et al., 2007; Kranz et al., 2009; Levitan et al., 2007), the response patterns can most likely not be extrapolated to other diazotrophs due to the fundamental differences described above. Several recent studies on N_2 fixers other than *Trichodesmium* have indeed yielded different CO_2 response patterns (e.g. Czerny et al., 2009; Fu et al., 2008; Garcia et al., 2013b; Wannicke et al., 2012), but very few investigations have compared multiple diazotroph species or strains under the same experimental conditions (Garcia et al., 2013b; Hutchins et al., 2013). To assess the diversity in CO_2 responses of N_2 fixers with very different physiology, we determined CO_2 effects on the single-celled *Cyanothece sp.* and two heterocystous species, *Nodularia spumigena* and the symbiotic *Calothrix rhizosoleniae* by growing them under present-day (380 μatm) and future pCO_2 levels (980 μatm). These results are then compared to literature data to relate the response patterns to specific physiological traits and structural characteristics of the different types of N_2 fixers.

2. Material and methods

2.1. Culture conditions

Cultures of *Calothrix rhizosoleniae* SC01, *Cyanothece sp.* ATCC51142 and *Nodularia spumigena* IOW-2000/1 were grown in semi-continuous dilute batch cultures at 25 °C and 150 μmol photons m^{-2} s^{-1} with a 12:12 h light:dark cycle, using 0.2-μm-filtered artificial seawater (YBCII medium; Chen et al., 1996). Salinity of the media was set to 33 for *Cyanothece* and *Calothrix* and 9 for *Nodularia* (measured with Autosal 8400B, Guildline). Cells were kept in exponential growth phase by regular dilution with culture medium. Cultures were grown in 1 L (*Cyanothece* and *Nodularia*) or 2 L (*Calothrix*) culture flasks, which were continuously bubbled with 0.2-μm-filtered air with pCO_2 levels of 380 and 980 μatm. As *Calothrix* cultures tended to form small, sinking aggregates, they were additionally placed on a shaker (ShakerX, Kuhner). Gas mixtures were generated with a gas flow controller (CGM 2000, MCZ Umwelttechnik), mixing pure CO_2 (Air Liquide Deutschland) and CO_2-free air (CO2RP280, Dominick Hunter). Culture media were equilibrated with the respective pCO_2 for at least 24 h before usage. Prior to experiments, cells were allowed to acclimate to the respective pCO_2 for two weeks (>5 divisions). Cultures with a pH drift of ≥ 0.09 compared to cell-free reference media were excluded from further analysis.

2.2. Carbonate chemistry

Total alkalinity (TA) was determined by duplicate potentiometric titration (TitroLine alpha plus, Schott Instruments) and calculation from linear Gran plots (Gran, 1952). Reproducibility was ± 5 μmol kg^{-1}. Samples for dissolved inorganic carbon (DIC) analysis were filtered through 0.2 μm cellulose acetate filters and measured colorimetrically (QuAAtro autoanalyzer, Seal, reproducibility ± 5 μmol kg^{-1}). Certified Reference Materials supplied by A. Dickson (Scripps Institution of Oceanography, USA) were used to correct for inaccuracies of TA and DIC measurements. Daily pH values of the media were measured potentiometrically on the NBS scale (pH meter pH3110, WTW, uncertainty 0.02 units). pCO_2 of the media was calculated from pH and DIC using CO2sys (Pierrot et al., 2006) with equilibrium constants K1 and K2 given by Mehrbach et al.

Table 1
Parameters of the carbonate system for each pCO_2 treatment acquired in daily measurements (pH) or at the start and end of growth curves (DIC and TA). Attained pCO_2 of the media was calculated from pH, DIC, phosphate concentration, temperature and salinity using CO2sys (Pierrot et al., 2006). Errors denote 1 SD (n ≥ 6).

	target pCO_2 [μatm]	pH (NBS)	TA [μmol kg^{-1}]	DIC [μmol kg^{-1}]	pCO_2 attained [μatm]
Cyanothece	380	8.20 ± 0.01	2356 ± 2	1969 ± 1	380 ± 9
	980	7.86 ± 0.02	2368 ± 13	2154 ± 10	974 ± 20
Nodularia	380	8.18 ± 0.02	1524 ± 28	1357 ± 12	391 ± 7
	980	7.80 ± 0.02	1515 ± 17	1399 ± 25	974 ± 39
Calothrix	380	8.25 ± 0.02	2348 ± 16	1950 ± 13	340 ± 30
	980	7.93 ± 0.03	2339 ± 28	2102 ± 18	832 ± 70

(1973), refit by Dickson and Millero (1987). Carbonate chemistry parameters for the different treatments are shown in Table 1.

2.3. Growth and elemental composition

Samples for determination of growth and elemental composition were generally taken between 1.5 and 3 h after the beginning of the photoperiod to account for changes due to the diurnal rhythm in cell metabolism. Cell densities and cell size of *Cyanothece* were determined using a Coulter counter (Multisizer III, Beckman Coulter). Cell densities of *Nodularia* cultures were determined using a Sedgwick Rafter Cell (Graticules Ltd.) and light microscope (Axiovert 200 M, Zeiss). Duplicate samples for chlorophyll *a* (chl *a*) determination were filtered onto cellulose-nitrate filters (Whatman) and immediately transferred to −80°C. Chl *a* was extracted in acetone (90%) for ~24 h and treated by ultrasound (~10 sec, Sonifier 250, Branson Ultrasonics). Chl *a* concentrations were determined fluorometrically (TD-700 Fluorometer, Turner Designs) and corrected for fluorescence of phycobilipigments (Holm-Hansen and Riemann, 1978). Calibration was performed measuring absorbance of a chl *a* standard (*Anacystis nidulans*, Sigma) spectrophotometrically (Spectronic Genesys 5, Milton Roy) according to Jeffrey and Humphrey (1975). Specific growth rates (μ) were calculated from daily increments in cell density (*Cyanothece* and *Nodularia*) or chl *a* concentrations (*Calothrix*) monitored over 6–7 days:

$$\mu[d^{-1}] = (\ln(c_1) - (\ln(c_0))/dt \quad (1)$$

where c_0 and c_1 are the initial and final cell density or chl *a* concentration and dt is the time interval between samplings. Duplicate samples for analysis of POC and PON were filtered onto pre-combusted (500 °C, 10 h) GF/F filters. Prior to analysis, samples were acidified with 200 μl ultrapure HCl (0.2 M) to remove all inorganic C. Particulate organic carbon and nitrogen (POC and PON) contents were measured with an elemental analyzer (EuroEA, Euro Vector). Daily production rates of POC and PON were obtained by multiplication of the respective elemental quotas and growth rates.

2.4. N_2 fixation

N_2 fixation rates were determined using the acetylene reduction assay (Capone, 1993). Samples were transferred to crimp vials and spiked with acetylene (20% of head space volume) followed by incubation for 24 h at acclimation light and temperature with continuous shaking to avoid aggregation of cells. DIC consumption during the course of the assay was found to be insignificant (<4%). The amount of acetylene reduced to ethylene was then measured with a gas chromatograph (5890 Series II Plus, Hewlett Packard), which was calibrated on a daily basis with an ethylene standard (Sigma-Aldrich). Solubility of acetylene in the aqueous phase was taken into account by applying the Bunsen coefficient for the respective temperature and salinities (Breitbarth et al., 2004).

2.5. Carbon fixation

C fixation rates were determined using ^{14}C fixation assays (Steemann-Nielsen, 1952). Samples were transferred to 25 ml vials with < 4% headspace and spiked with 20 μCi $H^{14}CO_3^-$ (1.576 GBq/mmol $NaH^{14}CO_3$ solution, Perkin Elmer), followed by incubation for 24 h at acclimation light and temperature with continuous shaking to minimize aggregation and sinking of cells. At the end of the incubation time period, samples were acidified with 6 N HCl (1 mL) to a final pH < 1, followed by degassing for ~3 days until all liquid had evaporated. Radioactivity of the samples was measured in a Packard Tri-Carb Liquid scintillation counter (GMI) between 24 and 48 h after addition of scintillation cocktail (UltimaGold AB, Packard). Counts were corrected for background radioactivity using samples that were acidified directly after addition of spike solution. The amount of C fixed during the assay (C_{fixed}) was calculated using the following equation:

$$C_{fixed} = (dpm_{sample} x [DIC] x 1.05)/dpm_{total} \quad (2)$$

where dpm_{sample} is the blank-corrected radioactivity of the acidified sample in dpm, [DIC] the respective concentration for each treatment, 1.05 the fractionation factor (Vogel et al., 1970) and dpm_{total} the total radioactivity of $H^{14}CO_3^-$ added to the sample. C fixation rates were normalized using chl *a* samples that were taken on the same day (*Calothrix*) and mean chl *a* cell quotas measured during the experiment (*Cyanothece* and *Nodularia*).

3. Results and discussion

The three species investigated in this study showed very different responses to elevated pCO_2. In the following paragraphs, we first discuss the response patterns for each species in view of its respective ecophysiology. Subsequently, literature data on other species are synthesized and response patterns are related to ecological and physiological characteristics of the different diazotrophs.

3.1. Species-specific response patterns reflect cellular mechanisms of nutrient housekeeping

In *Cyanothece*, growth rates were not affected by pCO_2 (*t*-test, $p > 0.05$, Fig. 1). In contrast, cell quotas of POC and PON increased to values more than twice as high at 980 compared to 380 μatm (*t*-test, $p < 0.05$, Table 2). Consequently, also production rates of POC and PON increased with pCO_2 (*t*-test, $p < 0.05$, Fig. 1). Since cell size was not significantly affected (*t*-test, $p > 0.05$, data not shown), cells must have strongly increased their C and N content per volume. Neither cellular chl *a* content nor the ratio of POC:PON were affected by pCO_2 (*t*-test, $p > 0.05$, Table 2). Also N_2 fixation was not significantly affected by pCO_2 (*t*-test, $p > 0.05$, Fig. 2). C fixation as determined in ^{14}C-fixation assays showed no significant difference between treatments due to high variability (*t*-test, $p > 0.05$, Fig. 2), yet indicated the same trend as POC production. Both methods, however, yielded different rates in absolute terms, with ^{14}C fixation being lower than POC production. As ^{14}C fixation samples were not filtered but acidified to degas all inorganic C, these values potentially also include dissolved organic C produced by the cells. Thus, it is even more puzzling why ^{14}C-based estimates were in fact lower than POC production rates. Part of this discrepancy could be explained by an overestimation of POC production due to diurnal variability in C quotas. The largest offset between methods was observed in *Cyanothece,* which has a strong diurnal cycle in cellular C and N quotas due to the temporal separation of N_2 fixation and photosynthesis. In the heterocystous *Nodularia*, less diurnal variability can be expected and accordingly, ^{14}C fixation and POC production were more consistent. This aspect can, however, not fully account for the observed offsets between methods. The acetylene reduction assay gives a measure of gross N_2 fixation, including any N that might be excreted from the cell subsequent to fixation. Absolute values of N_2 fixation and PON production are not directly comparable due to methodological issues, as pointed out previously by Mulholland and Capone (2001), and since the conversion factors from acetylene reduction to N_2 fixation have not been verified for the here tested species. Despite these uncertainties in absolute numbers, trends with pCO_2 can be interpreted for C as well as N. Furthermore, differences in trends between methods can give valuable indications of changes in N or C excretion. For *Cyanothece*, release of organic C and N measured in laboratory cultures was low (~2 and ~1% of fixed C and N, respectively; Benavides et al., 2013), suggesting a high nutrient retention potential. This is in line with the high variability in cellular contents (Table 2), which reflects effective storage

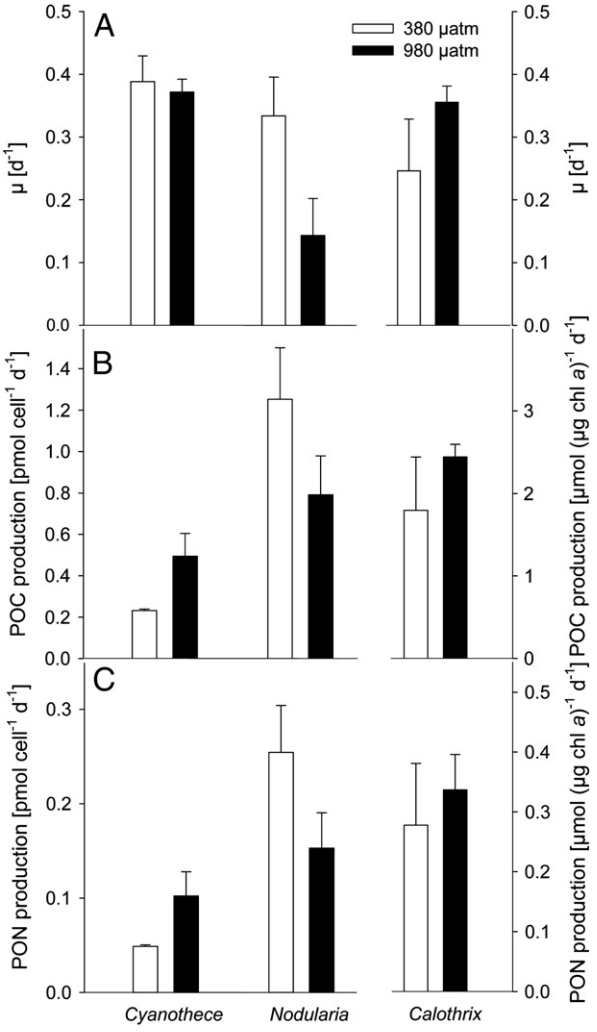

Fig. 1. Growth rates (A) and production rates of POC and PON (B & C) of *Cyanothece*, *Nodularia* and *Calothrix* grown at two different pCO_2 levels. Growth rates of *Cyanothece* and *Nodularia* were based on cell densities, growth rates of *Calothrix* were based on chl *a* concentrations. Errors denote 1 SD (n ≥ 3, except for POC & PON production in *Cyanothece* at 980 µatm with n = 2).

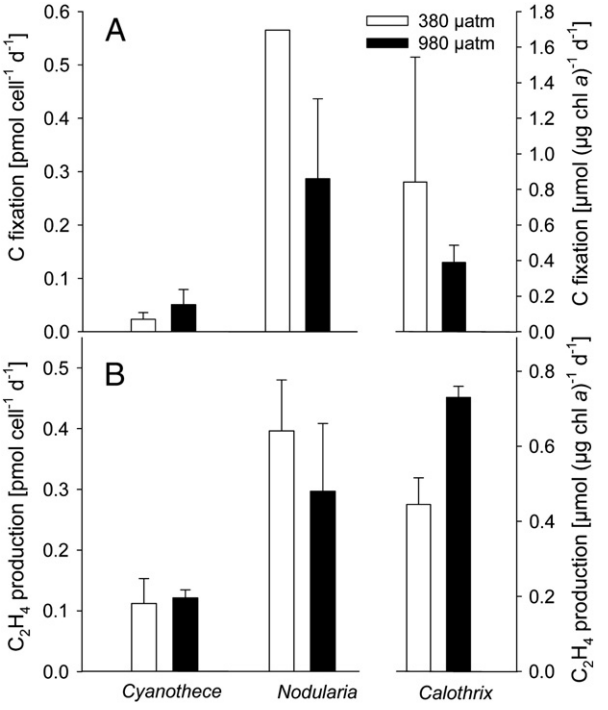

Fig. 2. C fixation (A) and ethylene (C_2H_4) production rates (B) of *Cyanothece*, *Nodularia* and *Calothrix* grown at two different pCO_2 levels. Errors denote 1 SD (n ≥ 3, except for C fixation in *Nodularia* at 380 µatm with n = 1).

mechanisms for C and N that are required due to the diurnal cycle of photosynthesis and N_2 fixation in this species. In each of the cells, fixed C is stored in glycogen granules and N can be directly used for synthesis of amino acids or cyanophycin, thus neither C nor N is subject to loss during intercellular transfer. The increase in POC with pCO_2 is consistent with the increase in glycogen contents found in a previous study testing effects of very high pCO_2 levels (10,000 and 80,000 µatm) on the same strain of *Cyanothece* (Stöckel et al., 2013).

In *Nodularia*, growth rates decreased significantly with increasing pCO_2 (*t*-test, $p < 0.05$, Fig. 1), while neither cellular chl *a* content nor POC and PON contents were significantly affected (*t*-test, $p > 0.05$, Table 2). POC:PON increased slightly with pCO_2 (*t*-test, $p < 0.05$, Table 2). POC and PON production generally showed a decreasing trend in response to elevated pCO_2, which was, however, only significant for PON production (*t*-test, $p < 0.05$, Fig. 1). The decrease in growth and production with increasing pCO_2 is in agreement with previous results by Czerny et al. (2009). In contrast, Karlberg and Wulff (2013) showed no effect, while Wannicke et al. (2012) found an increase in growth with pCO_2. In the latter study, however, cultures were limited by phosphate and were not continuously mixed. The negative effect of high pCO_2 on growth and production rates of *Nodularia* has been attributed to a detrimental effect of low pH on N transfer between cells along the filament (Czerny et al., 2009). This explanation has been debated, however, since transfer likely occurs via a continuous periplasm, preventing any direct contact of fixed N compounds with the outer medium (Flores et al., 2006; Wannicke et al., 2012). In our study, C:N ratios were increased only slightly with pCO_2 (Table 2), which also argues against any considerable obstruction of trans-cellular N transfer unless C transfer is equally inhibited. Aside from the uncertainty in absolute values derived by the two methods, the ratio of ^{14}C fixation to POC production was higher in *Nodularia* than in *Cyanothece* (Figs. 1 and 2),

Table 2
Cellular composition of *Cyanothece*, *Nodularia* and *Calothrix* grown at two different pCO_2 levels. Errors denote 1 SD (n ≥ 3, except for POC & PON in *Cyanothece* at 980 µatm with n = 2). n.d.: not determined.

	Target pCO_2 [µatm]	Chl *a* content [pg cell^{-1}]	POC content [pmol cell^{-1}]	PON content [pmol cell^{-1}]	POC : chl *a* [µmol µg^{-1}]	PON : chl *a* [µmol µg^{-1}]	POC:PON [mol:mol]
Cyanothece	380	0.036 ± 0.007	0.60 ± 0.06	0.13 ± 0.01	17.0 ± 4.6	3.6 ± 0.8	4.75 ± 0.23
	980	0.034 ± 0.007	1.27 ± 0.17	0.26 ± 0.04	42.5 ± 0.1	8.8 ± 0.3	4.85 ± 0.13
Nodularia	380	0.65 ± 0.12	4.08 ± 1.50	0.83 ± 0.29	6.4 ± 2.2	1.3 ± 0.5	4.92 ± 0.14
	980	0.53 ± 0.05	4.17 ± 0.36	0.81 ± 0.07	8.0 ± 1.2	1.5 ± 0.2	5.16 ± 0.04
Calothrix	380	n.d.	n.d.	n.d.	7.3 ± 0.2	1.1 ± 0.1	6.47 ± 0.13
	980	n.d.	n.d.	n.d.	6.9 ± 0.7	0.9 ± 0.1	6.64 ± 0.15

suggesting higher excretion of dissolved organic carbon in *Nodularia*. This is in line with previous studies showing significant excretion of fixed C and N by this species (Ploug et al., 2011; Wannicke et al., 2009). Regarding pCO_2 effects, while our C fixation data do not allow statistical evaluation due to the lack of replication for one of the treatments (Fig. 2), the decreasing trend in C fixation with elevated pCO_2 is consistent with the decrease in POC production (Figs. 1 and 2). Likewise, N_2 fixation was not significantly affected by pCO_2 (*t*-test, $p > 0.05$, Fig. 2), but mean values showed a decreasing trend, in line with PON production. The similarity in these trends suggests no appreciable effect of pCO_2 on excretion of C or N for *Nodularia*. This is in agreement with previous results showing no effect of pCO_2 on cell-specific production of mucinous substances and dissolved organic carbon (Endres et al., 2012).

In *Calothrix*, mean values of growth rates increased with pCO_2, yet the trend was not significant due to high standard deviations (*t*-test, $p > 0.05$, Fig. 1). POC and PON contents (relative to chl *a*) and production rates as well as the POC:PON ratio were not significantly affected (*t*-test, $p > 0.05$, Fig. 1, Table 2). In contrast, N_2 fixation showed a significant increase with pCO_2 (*t*-test, $p < 0.05$, Fig. 2). C fixation was not significantly different between pCO_2 treatments and was subject to high variability between replicates (*t*-test, $p > 0.05$, Fig. 2). Aggregation of filaments caused patchiness in our cultures, which precluded cell counts and resulted in deviations between triplicates for cellular composition, despite using large sample volumes. Results from bioassays are even more variable due to restrictions in sample volumes in the assays. Direct measurements of growth and cellular composition of *Calothrix* have to our knowledge not been performed previously and little is known about the physiology of *Calothrix* and other symbiotic cyanobacteria. While our study gives first indications of their CO_2 responses, it should be noted that the applicability to the natural environment is compromised by the fact that cells were cultured without the host. Cells in our cultures formed small aggregates, which may have caused microenvironments to slightly differ from the bulk medium. A similar situation in fact occurs in natural symbioses, where epibionts such as *Calothrix* are located in the boundary layer of the diatom cell, i.e. in a microenvironment shaped by the diatom's metabolism (Flynn et al., 2012). Moreover, the host cell seems to directly influence the cyanobacterial metabolism in symbioses, considerably increasing the N_2 fixation rates (Foster et al., 2011). While N_2 fixation rates measured in our study cannot be directly compared with previous data since cellular chl *a* quotas have not been determined, assuming a chl *a* quota similar to *Trichodesmium* the N_2 fixation rates obtained in our experiment (Fig. 2) are higher than those of free-living filaments measured in the field (0.17 fmol N per cell h^{-1}; Foster et al., 2011) and in a previous laboratory study (~1 to 4 μmol ethylene (mg chl *a*)$^{-1}$ h^{-1}; Foster et al., 2010). In fact, N_2 fixation rates are more similar to those of cells in symbiosis measured in field studies (70 fmol N per cell h^{-1}; Foster et al., 2011; 50 fmol N per cell^{-1} h^{-1} for *Richelia*, Carpenter et al., 1999), suggesting that the physiological status in our cultures may indeed be comparable to symbiotic *Calothrix* in their natural environment.

3.2. Ocean acidification responses across different N_2 fixers are highly variable

As outlined above, the three investigated species responded very differently to elevated pCO_2. A review of literature data showed that this large diversity in response patterns holds also for other species of N_2 fixing cyanobacteria (Table 3). To identify characteristics that might explain these differences, we grouped N_2 fixers according to their mechanisms for N_2 fixation (Table 3). This comparison revealed a considerably large variability of pCO_2 responses within groups and even species (Table 3). Part of this can be attributed to species- or strain-specific differences in CO_2 sensitivities (e.g. Hutchins et al., 2013; Langer et al., 2006; Schaum et al., 2013). It also has to be noted that growth conditions can significantly modulate the responses to elevated pCO_2 (e.g. Garcia et al., 2011; Kranz et al., 2010; Shi et al., 2012), which complicates linking response patterns to specific traits. Lastly, the low number of studies on certain groups does not permit deducing clear statements on their CO_2 sensitivity.

Especially for heterocystous species, there is little data with often contrasting results, indicating stimulation in N_2 fixation for *Calothrix* and negative as well as positive responses towards elevated pCO_2 for *Nodularia* (Table 3). The diazocystous *Trichodesmium*, however, has been extensively studied and the data are more coherent, showing a positive response of N_2 fixation in most cases (Table 3). Also single-celled species responded positively to elevated pCO_2 in many of the studies, yet the magnitude of effects was generally smaller than in *Trichodesmium*. For instance, growth was significantly increased in about half of the observations on *Trichodesmium* but was not altered in most observations on single-celled species (Table 3). Also, in cases where an increase in N_2 fixation was observed, the magnitude of effects ranged between ~35 and ~140% in *Trichodesmium*, while for single-celled species it ranged only between ~20 and 45% (Table 3). Regarding cellular composition and production of POC and PON, data are scarce for single-celled species (Table 3). While we observed a strong increase in C and N quotas for *Cyanothece*, no such changes were observed in the only other study investigating C quota of *Crocosphaera* (Fu et al., 2008; Table 3). For understanding the variable responses to rising pCO_2, it thus seems insufficient to consider only the functional diversity in modes of N_2 fixation. Looking at the present-day growth conditions of a species may also provide hints about its sensitivity towards ocean acidification.

3.3. CO_2 responses are dependent on ecological niches

The species investigated in our study grow in different habitats, which strongly diverge with respect to carbonate chemistry. Compared to the relatively stable conditions experienced in open-ocean environments by diazotrophs such as *Cyanothece*, species in the Baltic Sea such as *Nodularia* are subject to highly variable carbonate chemistry over time. This is due to the low alkalinity as well as high biological activity of dense cyanobacterial blooms inducing strong seasonal variability (Thomas and Schneider, 1999). Regarding effects of increased CO_2 supply on the three species investigated here, adaptation of their carbon concentrating mechanisms (CCM) to the respective habitats might thus cause different CO_2 sensitivities. In surface scums formed by summer blooms of *Nodularia* and *Aphanizomenon*, pH can reach values as high as 9 during illumination (Ploug, 2008). Thus, one could explain the negative response of *Nodularia* to pCO_2 levels exceeding 380 μatm by assuming that their mode of CCM and pH homeostasis has evolved to function optimally under low pCO_2/high pH. In fact, Czerny et al. (2009) hypothesized maximal growth rates even below a pCO_2 of 150 μatm. In the case of symbiotic *Calothrix*, carbonate chemistry in the immediate surroundings of the cell is modulated by the diatom host, which can significantly alter CO_2 concentrations and pH by respiration and photosynthesis. Since diurnal variability within the microenvironment of photoautotrophs is directly dependent on their size (Flynn et al., 2012), variability can be expected to be larger for a symbiont on a diatom chain than for a single cyanobacterial cell or filament, though by far not as large as in surface scums of *Nodularia* in the Baltic. In our experiment, *Calothrix* did not show negative responses as observed in *Nodularia*, but tended to respond positively to elevated pCO_2 like the single-celled *Cyanothece* (Table 3). Thus, while *Nodularia* and *Calothrix* are both heterocystous, adaptation of their CCM and/or their mode of pH homeostasis to different environmental conditions might explain the differences in pCO_2 responses.

3.4. Magnitudes of CO_2 responses can be attributed to cellular energy demand

Due to the strong interdependence of different physiological pathways in the cell, several other factors apart from carbonate chemistry

Table 3
Overview of literature data on pCO_2 responses of different N_2 fixing cyanobacteria. Arrows indicate direction of trend with increasing pCO_2; numbers in brackets indicate percent change relative to low pCO_2 level calculated from original data; significance was taken from the respective publications. For comparison reasons, only data for pCO_2 levels closest to our treatments and normalized to cell or chl *a* (where available) are shown. For studies with varying nutrient conditions, data from replete conditions are shown, unless stated otherwise. Light intensity is given in µmol photons m^{-2} s^{-1}.

	Genus	Species/strain	pCO_2 [µatm]	Light intensity	Growth	POC content	PON content	POC:PON	N_2 fixation	O_2 evol. or C fix.	Reference
Single cells	*Crocosphaera*	*watsonii* WH8501	380 vs 750	80	↔	↔		↔	↑ (+40%)	↑ (+20%)[2]	(Fu et al., 2008)
			280 vs 750	120					↑ (+20%)		(Hutchins et al., 2013)
		watsonii WH0003	280 vs 750	120					↔		(Hutchins et al., 2013)
			190 vs 810	150	↑ (+60%)				↑ (+45%)	↑ (+60%)[2]	(Garcia et al., 2013a)
		watsonii WH0402	380 vs 750	100	↔				↔	↑ (+35%)[2]	(Garcia et al., 2013b)
		watsonii WH0401	380 vs 750	100	↔				↔	↑ (+17%)[2]	(Garcia et al., 2013b)
			280 vs 750	120					↑ (+35%)		(Hutchins et al., 2013)
	Cyanothece	*sp.* ATCC51142	380 vs 980	150	↔	↑ (+110%)	↑ (+100%)	↔	↔	↔[2]	(This study)
Diazocytes	*Trichodesmium*	*erythraeum* IMS101	380 vs 850	150	↔	↓ (−60%)	↓ (−60%)	↔	↔		(Barcelos é Ramos et al., 2007)
			380 vs 750	100				↔	↑ (+35%)	↑ (+40%)[2]	(Hutchins et al., 2007)
			400 vs 900	80–120	↑ (+50%)			↑ (+8%)	↑ (+140%)	↔[1]	(Levitan et al., 2007)
			370 vs 1000	150	↔	↑ (+30%)	↑ (+30%)	↔			(Kranz et al., 2009)
			150 vs 900	200	↑ (+11%)	↑ (+9%)	↑ (+20%)	↓ (−8%)	↑ (+110%)	↔[1]	(Kranz et al., 2010)
			380 vs 750	100	↑ (+30%)	↔	↔		↑ (+70%)	↑ (+60%)[2]	(Garcia et al., 2011)
			380 vs 750	90	↓ (−20%)			↔	↓ (−50%)	↔[2]	(Shi et al., 2012)[3]
		erythraeum GBRTRLI101	380 vs 750	100				↔	↑ (+45%)	↑ (+30%)[2]	(Hutchins et al., 2007)
			380 vs 750	220	↔				↔	↑ (+17%)[2]	(Garcia et al., 2011)
			370 vs 750	120					↑ (+50%)		(Hutchins et al., 2013)
		erythraeum KO4-20	280 vs 750	120					↑ (+35%)		(Hutchins et al., 2013)
		contortum 2174	280 vs 750	120					↑ (+30%)		(Hutchins et al., 2013)
		thiebautii H9-4	280 vs 750	120					↔		(Hutchins et al., 2013)
		thiebautii	380 vs 800	?				↔	↑ (+55%)	↔[2]	(Lomas et al., 2012)[4]
Heterocysts	*Nodularia*	*spumigena* IOW 2000/1	380 vs 800	85	↓ (−30%)	↑ (+20%)	↔	↑ (+17%)	↓ (−15%)		(Czerny et al., 2009)
			380 vs 980	150	↓ (−60%)	↔	↔	↑ (+5%)	↔		(This study)
		spumigena	~400 vs ~500	200	↑ (+40%)	↓	↓	↔	↑ (+35%)	↑ (+20%)[2]	(Wannicke et al., 2012)[5]
	Calothrix	*rhizosoleniae* SC01	380 vs 980	150	↔	↔	↔	↔	↑ (+60%)	↔[2]	(This study)

[1] O_2 evolution.
[2] C fixation.
[3] Low iron.
[4] Field incubations.
[5] Low phosphate.

can affect C acquisition and thus alter the overall CO_2 responses of phytoplankton. Studies on the underlying mechanisms of CO_2 responses suggested a reallocation of energy between the CCM and N_2 fixation in *Trichodesmium* (Kranz et al., 2009, 2010). This was confirmed by a strong modulation of CO_2 effects by light intensity (Kranz et al., 2010), suggesting that the degree of energy limitation plays an important role in controlling CO_2 responses in this species. A dependence of CO_2 effects on energy limitation has also been suggested for *Crocosphaera* in a study on combined effects of pCO_2 and phosphorus limitation, which linked the magnitude of pCO_2 effects to ATP pool size (Garcia et al., 2013a). Daily synthesis of nitrogenase and the photosynthetic and respiratory protein complexes is supposed to consume a significant fraction of cellular resource and energy costs in *Trichodesmium* (Brown et al., 2008). For single-celled species such as *Crocosphaera* and *Cyanothece*, it can be assumed that the day-night separation of photosynthesis and N_2 fixation similarly imposes high energy demands for the turnover of enzymes and storage products. Thus, while single-celled species may respond positively to elevated pCO_2 due to the lowered energy demand of the CCM, just as proposed for *Trichodesmium*, the difference in magnitudes of responses might be associated to differences in energy limitation under the experimental conditions. While *Crocosphaera* occurs with maximum abundance at ~35 – 60 m depth (Hewson et al., 2009; Montoya et al., 2004), *Trichodesmium* is known to form surface blooms (Capone et al., 1997). One could therefore speculate that *Trichodesmium* experienced relatively stronger energy limitation under the light conditions typically applied in the experiments (Table 3). Not only the overall magnitude of CO_2 effects but also the relative sensitivity of C and N_2 fixation differed between *Trichodesmium* and single-celled species: In *Trichodesmium*, N_2 fixation was strongly increased in many of the studies (up to 140%; Table 3), while C fixation/O_2 evolution were increased only in part of the studies and to a lesser extent (up to 60%; Table 3). In contrast, single-celled species showed an increase in N_2 fixation only in half of the observations (reaching up to 45%), while C fixation was significantly increased (up to 60%) in all except our study (Table 3). This pattern suggests that the reallocation of energy from C acquisition to N_2 fixation in the single-celled species was not as pronounced as in *Trichodesmium*. The physiology of C acquisition in *Crocosphaera* or *Cyanothece* has not been characterized to date. However, based on the differences in cell/filament size and thus diffusive boundary layers, it could be expected that small, single-celled species can cover more of their C demand by CO_2 diffusion than the large filaments/aggregates of *Trichodesmium* (Edwards et al., 2011; Finkel et al., 2010; Wolf-Gladrow and Riebesell, 1997). If cells rely to a large extent on diffusive CO_2 supply rather than active uptake of HCO_3^-, elevated CO_2 concentrations will directly increase C influx with supposedly little effect on the cellular energy budget, and consequently also little feedback on N_2 fixation.

In heterocystous species such as *Nodularia*, overall energy demands connected to N assimilation might be lower than in single-celled species or *Trichodesmium*, since daily synthesis and degradation of nitrogenase as well as high turn-over of storage products are not necessary. The N transfer between cells also seems to be more efficient in *Nodularia* (excretion up to 33%; Ploug et al., 2011) than in *Trichodesmium* (excretion up to 80%; Mulholland, 2007), which is in line with transfer being conducted directly between adjoining cells rather than extracellularly (Flores et al., 2006). If the magnitude of CO_2 responses is directly dependent on the level of energy limitation, as proposed for *Trichodesmium* (Kranz et al., 2010), the lower energy demands for N assimilation may explain why *Nodularia* did not respond positively to high pCO_2 in our study. In line with this, the positive CO_2 responses observed in *Nodularia* by Wannicke et al. (2012) might reflect energy constraints induced by phosphate limitation in that experiment. However, neither the costs nor the regulation of C acquisition in *Nodularia* have been investigated to date. Knowledge on the physiology of *Calothrix* is even more limited and energy demands are most likely highly variable depending on whether cells occur in free-living filaments or in symbioses.

3.5. Benefits from CO_2 concentration outbalance negative pH effects in most, but not all N_2 fixers

For most of the N_2 fixers tested to date, a benefit from either the direct effect of CO_2 supply or the energy saved from down-regulation of CCM activity seems to allow positive responses to ocean acidification. While differences in the magnitude of these CO_2 effects could be attributed to cellular energy limitation, negative responses, as observed in *Nodularia*, cannot be explained by the costs of C acquisition. The concurrent decrease in seawater pH under ocean acidification affects many cellular processes, such as enzyme functioning, transport processes and ion balance, as well as speciation of nutrients (e.g. Hinga, 2002). In *Trichodesmium* grown under low iron concentrations, low pH has also been shown to negatively affect nitrogenase efficiency (Shi et al., 2012). As nitrogenase is a highly conserved protein complex (Zehr et al., 2003), this pH effect on the enzyme seems likely to be relevant also for other diazotrophs. In previous DIC manipulation experiments with *Trichodesmium* under nutrient replete conditions, this negative effect was apparently outbalanced by the strong positive effect of high CO_2 concentrations. In *Nodularia*, on the other hand, positive effects of high pCO_2 seemed to be attenuated, possibly due to adaptation to low pCO_2 levels and a lower energy demand of N assimilation, which allowed negative effects of the pH to dominate. For a better understanding of these opposing effects, both factors should be tested independently in a setup with uncoupled carbonate chemistry (e.g. Bach et al., 2013).

3.6. Biogeochemical implications differ between ecosystems and depend on ecological interactions

Concerning implications on the ecosystem level, the wide variety in pCO_2 responses of N_2 fixers is likely to affect community composition in future oceans (Hutchins et al., 2013). Since N_2 fixing species differ with regard to important ecological and physiological characteristics, such as susceptibility to grazing, sinking and nutrient release, these species shifts will further impact on biogeochemical cycles of C and N (Sohm et al., 2011). For instance, the increase in POC and PON production rates at high pCO_2 observed in *Cyanothece* would increase C and N input into the photic zone. The possible increase in N_2 fixation rates by single-celled species as well as *Calothrix* adds on to the increase in N_2 fixation projected for *Trichodesmium*. The fate of fixed N within the ecosystem, however, is likely to differ between diazotroph species (Thompson and Zehr, 2013). While *Trichodesmium* is hardly grazed and releases large amounts of fixed N that is directly available to other phytoplankton (e.g. Mulholland, 2007), in *Cyanothece* fixed N seems to be efficiently stored and may thus be transferred more directly to higher trophic levels. In *Calothrix*, the bulk of fixed N is transferred to the diatom host cells (Foster et al., 2011). While diazotrophs are often neutrally buoyant, diatoms sink relatively fast due to their dense silica shells. A possible increase in DDA blooms due to the stimulation in N_2 fixation would thus have profound impacts on the global C cycle, fuelling the biological pump and therewith CO_2 sequestration (Karl et al., 2012; Subramaniam et al., 2008).

In contrast to the stimulating effect of high pCO_2 on open-ocean symbiotic and single-celled species, our study as well as Czerny et al. (2009) showed decreasing growth and production rates for the Baltic *Nodularia*. Since results from different studies on this species are conflictive (cf. Karlberg and Wulff, 2013; Wannicke et al., 2012), further investigations are necessary before making conclusive predictions of ecosystem responses, which should also include other important Baltic diazotrophs, such as *Aphanizomenon*. *Nodularia* and *Aphanizomenon* frequently co-occur in cyanobacterial blooms and their relative abundance has been shown to be strongly dependent on environmental perturbations (Stal et al., 2003). Species interactions and possible differences in CO_2 sensitivity could change community composition and therewith toxicity of future blooms, as *Nodularia* produces the hepatotoxin

nodularin (Karlberg and Wulff, 2013; Sivonen et al., 1989). Aggregates and surface scums, such as those formed by *Nodularia*, are usually heavily colonized by heterotrophic bacteria and play an important role in promoting regenerated production due to the excretion of inorganic C and N (Ploug et al., 2011; Simon et al., 2002). A decrease in *Nodularia* productivity may therefore have profound impacts on Baltic nutrient fluxes, depending on the response of cyanobacteria with similar ecological function such as *Aphanizomenon*.

4. Conclusions

In summary, the three species of functionally different N_2 fixers investigated in this study responded differently to elevated pCO_2, showing enhanced, decreased as well as unaltered growth and production rates. This confirms the large diversity of pCO_2 responses found among previous studies and suggests that the deviations between studies were not merely due to discrepancies in experimental conditions. Our review showed that while CO_2 effects on *Trichodesmium* have been extensively studied, we cannot extrapolate these responses to other diazotrophs. Based on results from this and previous studies, we were able to draw connections between response patterns and regional differences in carbonate chemistry as well as species-specific differences in energy requirements. These interrelations yet need to be confirmed by physiological studies to enable more robust predictions of response patterns.

The observed differences in CO_2 sensitivities will impact on species distribution and biogeochemical cycles and should therefore be considered in ecosystem and global models estimating climate feedback effects. In areas of strong competition between N_2 fixers, contrasting responses to rising pCO_2 can lead to considerable regional changes, which should be considered in small-scale, regional models. In addition to *Trichodesmium*, also single-celled and symbiotic open-ocean species were found to respond positively to high pCO_2. Despite species-specific differences observed in the magnitude of responses, our study thus confirms the predictions of an increase in N_2 fixation with ocean acidification on a global scale. In contrast, N input to the Baltic Sea might decrease due to potentially lowered productivity of *Nodularia*.

Large uncertainty remains with regard to the poorly characterized UCYN-A cyanobacteria, which are increasingly recognized to have a high biogeochemical importance. Their pCO_2 responses and underlying mechanisms are most probably very different from the diazotrophs studied so far, as they do not fix C and are thus unlikely to react to CO_2 concentrations directly, but may instead be more influenced by pH and/or their possible host species. The physiology and abundance of this group of cyanobacteria as well as the response of symbionts within the microenvironment of their host clearly need further investigation before CO_2-dependent alterations in the N cycle can be predicted.

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