



Atmospheric CO₂ availability induces varying responses in net photosynthesis, toxin production and N₂ fixation rates in heterocystous filamentous Cyanobacteria (*Nostoc* and *Nodularia*)

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Received: 21 April 2020 / Accepted: 3 February 2021 / Published online: 27 February 2021
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Abstract

Heterocystous Cyanobacteria of the genus *Nodularia* form major blooms in brackish waters, while terrestrial *Nostoc* species occur worldwide, often associated in biological soil crusts. Both genera, by virtue of their ability to fix N₂ and conduct oxygenic photosynthesis, contribute significantly to global primary productivity. Select *Nostoc* and *Nodularia* species produce the hepatotoxin nodularin and whether its production will change under climate change conditions needs to be assessed. In light of this, the effects of elevated atmospheric CO₂ availability on growth, carbon and N₂ fixation as well as nodularin production were investigated in toxin and non-toxin producing species of both genera. Results highlighted the following:

- Biomass and volume specific biological nitrogen fixation (BNF) rates were respectively almost six and 17 fold higher in the aquatic *Nodularia* species compared to the terrestrial *Nostoc* species tested, under elevated CO₂ conditions.
- There was a direct correlation between elevated CO₂ and decreased dry weight specific cellular nodularin content in a diazotrophically grown terrestrial *Nostoc* species, and the aquatic *Nodularia* species, regardless of nitrogen availability.
- Elevated atmospheric CO₂ levels were correlated to a reduction in biomass specific BNF rates in non-toxic *Nodularia* species.
- Nodularin producers exhibited stronger stimulation of net photosynthesis rates (NP) and growth (more positive Cohen's d) and less stimulation of dark respiration and BNF per volume compared to non-nodularin producers under elevated CO₂ levels.

This study is the first to provide information on NP and nodularin production under elevated atmospheric CO₂ levels for *Nodularia* and *Nostoc* species under nitrogen replete and diazotrophic conditions.

Keywords Climate change · Nitrogen fixation · *Nodularia* · Nodularin · *Nostoc* · Photosynthesis

Abbreviations

BNF Biological nitrogen fixation
HC High CO₂ (2000 ppm)
LC Low CO₂ (~440 ppm)
POC Particulate organic carbon

PON Particulate organic nitrogen
NP Net photosynthesis
NPP Net primary production
CCM Carbon concentrating mechanism

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Introduction

Cyanobacteria, in their role as primary producers, form an essential part of the global C and N cycles, both in terrestrial and aquatic environments (Visser et al. 2016; Elbert et al. 2012). The process of oxygenic photosynthesis, whereby energy from the sun is used to reduce inorganic carbon with the accompanying oxidation of water, is thought to

have evolved during the Archean era when there was no free oxygen in the Earth's atmosphere (Lyons et al. 2014). The enzyme catalysing CO₂ fixation in Cyanobacteria and modern-day C3 plants is ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), thought to be the most abundant enzyme on Earth. Rubisco binds CO₂ and generates 2 molecules of 3-phosphoglycerate (3PGA) which is further processed in the Calvin-Benson-Bassham (CBB) cycle to produce ribulose-1,5-bisphosphate and glutamate. In order to reduce undesirable oxygenase activity, Cyanobacteria have evolved the carbon concentrating mechanism (CCM) to increase the effective concentration of CO₂ around the Rubisco active site by up to 1000-fold (Price 2011). CO₂ diffuses freely into the cell and is converted to bicarbonate in an NADPH-dependent reaction. Most Cyanobacteria sequenced to date carry the high flux, low affinity CO₂ converting enzyme, NDH-I₄, as well as the low flux, high affinity NDH-I₃ variant. Uptake of bicarbonate from the surrounding liquid requires an investment in energy and the synthesis of specific transporters. Two sodium-dependent symporters, BicA (high flux, low affinity) and SbtA (low flux, high affinity) bicarbonate transporters occur occasionally together with the BCT1 high affinity low flux transporter, found in almost all Cyanobacteria investigated to date on the cell membrane (Burnap et al. 2015; Visser et al. 2016). The presence of BicA provides aquatic Cyanobacterial species a growth advantage under elevated levels of HCO₃⁻ availability (Sandrini et al. 2014). Oxygenic photosynthetic organisms that rely on the construction of a carbon concentrating mechanism (CCM) are thought to be sensitive to changes in pCO₂ (e.g. Raven et al. 1991; Rost et al. 2003; Price 2011; Shi et al. 2012; Raven et al. 2017). The plasticity of the CCM to elevated levels of atmospheric CO₂ was found to be high in Cyanobacteria when compared to the more recently evolved haplophytes and diatoms (Van de Waal et al. 2019). This phenotypic plasticity in carbon fixation was demonstrated on *Microcystis* grown under conditions of elevated CO₂ (Ji et al. 2020). The maximum CO₂ uptake rate of *Microcystis* grown at 1000 ppm CO₂ was increased 1.5–1.8 times compared to the low CO₂ control cultures, suggesting that elevated CO₂ conditions may stimulate Cyanobacterial bloom growth (Ji et al. 2020). Furthermore, by reducing the levels of dissolved CO₂ and increasing the pH in dense blooms, Cyanobacterial species succession is thought to be driven towards strains with a more efficient carbon concentrating mechanisms (Lines and Beardall 2018).

Globally, an increase in phytoplankton blooms, including Cyanobacterial harmful algal blooms, has been recorded since the 1980's (Ho et al. 2019). While the reasons for the observed increase is unclear, temperature, elevated atmospheric CO₂ levels and eutrophication especially of the freshwater lakes are potential drivers of this phenomenon. Approximately a third of all anthropogenic CO₂ released

dissolves in the oceans, reducing the pH by increasing the partial pressure of CO₂, accompanied by a smaller relative increase in HCO₃⁻ and a decrease in CO₃²⁻ (Sabine et al. 2004; Raven et al. 2017). Although the speciation of dissolved inorganic carbon is directly linked to pH, how changes in their balance affects Cyanobacterial bloom occurrence and toxicity is unclear (Raven et al. 2020). The increase in growth rate observed for the marine, non-heterocystous, filamentous diazotrophic Cyanobacterium, *Trichodesmium*, grown at 900 ppm CO₂ was ascribed to down regulation of the CCM, thereby reducing the energy demands on the cell (Kranz et al. 2011). Under Fe-limiting conditions, decreasing the medium pH reduced N₂ fixation rates in *Trichodesmium*, with the reduced N₂ fixation rates corresponding to reduced nitrogenase efficiency at lower pH (Kranz et al. 2011). Exposing cultures of the freshwater diazotroph, *Nostoc muscorum*, to raised HCO₃⁻ concentrations under diazotrophic conditions resulted in enhanced growth, O₂ and pigment production and nitrogenase activities (Bhargava et al. 2013). The brackish diazotroph, *Nodularia spumigena* sp. KAC12, when grown at elevated CO₂ of 960 ppm, demonstrated increased photochemical yield after 5 days exposure (Karlberg and Wulff 2013), suggesting higher potential net primary productivity rates. *Nodularia spumigena* CCY9414, grown under elevated CO₂ conditions (548 ppm), exhibited increased C fixation rates compared to control cultures, with increased carbon to nitrogen (POC:PON) and nitrogen to phosphate ratios recorded (Wannicke et al. 2012). Only a slight increase was observed in the C:N ratios in three Cyanobacterial cultures grown at elevated CO₂ (~900 ppm) in continuous culture in bubble reactors, namely *Cyanothece* sp. ATCC51142, *Nodularia spumigena* IOW-2000/1 and *Calothrix rhizosoleniae* sp. SC01 (Eichner et al. 2014). This study emphasised the need to generate more data on the effects of elevated CO₂ levels on Cyanobacterial BNF, and highlighted the diversity in observed responses of marine Cyanobacterial species to elevated atmospheric CO₂. Wannicke et al. (2018b), in their metadata study, found indications that ocean acidification would benefit BNF in the future ocean. They also drew attention to the fact that these studies were mostly conducted on only two species, the filamentous *Trichodesmium* and unicellular *Crocospaera*. Very few studies were published on filamentous heterocystous *Nodularia*, *Calothrix* and *Anabaena* (alias: *Dichlosperrum*) species (reviewed by Wannicke et al. 2018b). A more recent study suggested that growth of the diazotrophic *Dolichospermum circinale* might benefit from increased CO₂ levels of 1700 ppm (Symes and van Ogtrop 2019).

Studies investigating the effect of climate change on filamentous diazotrophic Cyanobacteria in terrestrial habitats are rare too. Terrestrial surfaces are often inhabited by cryptogamic covers, including Cyanobacteria that contribute

a significant amount to global net primary productivity (Elbert et al. 2012). Specifically, it is estimated that N₂ fixation by cryptogammic covers may account for almost half of biological nitrogen fixation on land, ~49 Tg per year (Elbert et al. 2012). Biological soil crusts showed a decrease in Cyanobacterial abundance when grown under elevated atmospheric CO₂ for 10 years, suggesting a negative impact of climate change on arid soil crusts (Steven et al. 2012). The ability of Cyanobacterial soil crusts to increase net primary production under high CO₂ (HC) exposure was shown to be dependent on water availability (Lane et al. 2013). This was in agreement with previous research demonstrating that terrestrial *Nostoc flagelliforme* exhibited its highest relative growth rate under conditions of high CO₂ (1500 ppm) in moist conditions when compared to mats grown at 350 ppm CO₂ (Gao and Yu 2000). Rodriguez-Caballero et al. (2018) suggested that dryland soil crusts are under threat due to anthropogenically induced climate change. Their projected loss of 25–40% cryptogammic coverage will result in reduced microbial contributions to nitrogen cycling and soil surface stabilisation. Additionally, little information exists as to how Cyanobacterial biocrusts specifically, will respond to increasing atmospheric CO₂ levels (Reed et al. 2016).

Given the evolutionary history of Cyanobacteria already existing under raised CO₂ levels, researchers (Gehring and Wannicke 2014; Sandrini et al. 2016; Visser et al. 2016; Buratti et al. 2017) have voiced concerns for increased Cyanobacterial bloom occurrence and toxin production under the elevated levels of CO₂ proposed by current climate change scenarios, particularly in eutrophic waters (Ma et al. 2019). Especially toxin producing Cyanobacteria capable of fixing atmospheric N₂ would offer a potential threat to human safety, as they could thrive in otherwise nitrogen-limited habitats (O'Neil et al. 2012). The most commonly occurring Cyanobacterial toxins are microcystin and nodularin, both strong protein phosphatase inhibitors, capable of inducing extensive hepatocellular bleeding and collapse in exposed individuals and animals (Gehring 2004; Ibelings et al. 2015; Buratti et al. 2017). Microcystin and nodularin are synthesized by non-ribosomal peptide synthetases (Dittmann et al. 2001; Moffit and Neilan 2004) for which the control mechanisms remain largely unknown. The levels of toxin production within Cyanobacterial blooms is largely determined by several abiotic factors such as light intensity and quality, pH and nutrient availability (Reviewed by Gehring and Wannicke 2014; Visser et al. 2016; Buratti et al. 2017). Raised temperatures and elevated CO₂ levels in the range of those proposed under climate change, are linked to increased primary production (Paerl and Huisman 2009) and toxin production by Cyanobacteria (El-Shehawey et al. 2012; Kleinteich et al. 2012). Increased production of the secondary metabolite, microcystin, is linked to maintaining the C:N balance in the cell in the non-diazotrophic

Microcystis aeruginosa (Downing et al. 2005), particularly when N uptake exceeds the relative growth rate. Elevated CO₂ levels have the capacity to affect the community composition and toxicity of *Microcystis* blooms significantly (Liu et al. 2016; Van De Waal et al. 2011; Sandrini et al. 2016; Buratti et al. 2017). Microcystin synthesis requires active photosynthesis (Sevilla et al. 2012) and, like nodularin synthesis, is regulated by the global N uptake regulator, NtcA, supporting the proposed importance of the C:N balance on toxin production (Neilan et al. 2013). This agrees with observed anthropogenically induced alterations in environmental N:P ratios, resulting in the appearance of Cyanobacterial blooms (Beverdort et al. 2013) and increased toxin production (Horst et al. 2014). Inorganic nitrogen limitation was thought to induce a shift to N₂ fixing, diazotrophic Cyanobacteria, thereby increasing organic N availability and a subsequent increase in toxin production (Posch et al. 2012; Gehring and Wannicke 2014). Recent investigations of bloom dynamics in Lake Müggelsee suggest that the predominant Cyanobacterial diazotrophs, *Aphanizomenon* sp. and *Anabaena* sp (*Dichlosperrum* sp.), do not proportionally increase in numbers relative to non-nitrogen producers under conditions of reduced N availability (Shatwell and Köhler 2019). The changes in Ci availability, nitrogen fixation rates and potential cyanotoxin production levels were not reported. Transcription of the *nda* cluster in *Nodularia spumigena* AV1 was found to be altered in response to changes in ammonia and phosphate availability, however, the levels of intra- and extracellular nodularin were not significantly altered (Jonasson et al. 2008). Production of cylindrospermopsin and microcystin is thought to be constitutive, with cell cyanotoxin quotas being relatively fixed (Orr et al. 2018; Pierangelini et al. 2015). Orr et al. (2018) furthermore argued that toxicity is not affected through any stimulatory or trigger effect on the toxin production pathway itself, but via changes in rates of cell division and growth of different strains with genetically different cyanotoxin cell quotas. If the Cyanobacterial specific cyanotoxin rate matches the specific cell division rate, the overall cell cyanotoxin remains fixed. Dense Cyanobacterial blooms require excessive CO₂ to support their continued growth (Paerl and Huisman 2009) with CO₂ availability often limiting bloom growth, a restriction that could be removed under increased atmospheric CO₂ levels. Only aquatic Cyanobacteria carrying the high flux, low affinity BicA HCO₃⁻ receptor were able to benefit from elevated CO₂ levels and increase their growth rates (Sandrini et al. 2015; 2016; Visser et al. 2016).

Most studies on elevated CO₂ effects reported for toxin producing Cyanobacteria have focused on the production of the heptapeptide toxin, microcystin, in freshwater unicellular non-diazotrophic *Microcystis aeruginosa* species. The microcystin content of *Microcystis aeruginosa* HUB 5-2-4 grown at elevated CO₂ was raised, while growth rates

were kept constant (Van de Waal et al. 2009). Sandrini et al. (2015) reported that the shift of *Microcystis aeruginosa* PCC 7806 from 200 ppm $p\text{CO}_2$ to 1450 ppm $p\text{CO}_2$ in a continuous culture, resulted in a ~ 2.7 -fold increase of Cyanobacterial biomass and ~ 2.5 -fold elevation in microcystin per cell. Moreover, at high $p\text{CO}_2$, gene expression of the high flux, low affinity BicA HCO_3^- receptor was down-regulated and cells shifted to CO_2 and low-affinity, high flux receptors for bicarbonate uptake. Interestingly, the expression of the *mcy* genes involved in microcystin synthesis remained constant, suggesting additional regulatory steps are involved in toxin synthesis under elevated CO_2 conditions. Studies investigating the competition of microcystin and non-microcystin producing strains of *Microcystis* at low and elevated $p\text{CO}_2$ levels found that non-toxic strains outcompete toxic strains under conditions of low light and high CO_2 availability (Van De Waal et al. 2011; Yu et al. 2015). On the other hand, toxin-producing strains display a better fitness under growth-limiting conditions suggesting that the benefit of producing the toxin outweighs its costs under unfavourable conditions (Briand et al. 2008; Van De Waal et al. 2011).

To our knowledge, there is no peer-reviewed publication concerning the effect of elevated CO_2 on nodularin production in Cyanobacteria. This study is directed at studying diazotrophic Cyanobacterial species from both terrestrial and aquatic environments to investigate the effect of elevated CO_2 levels on net photosynthesis, toxin production, growth and N_2 fixation rates in a multiple matrix approach. To do so, seven different species were chosen of which three are able to produce the toxin nodularin and four are non-nodularin producers. Finally, we analysed the data set gained in this study by applying weighted mean effect sizes to test the hypothesis that Cyanobacteria react differently towards elevated CO_2 depending on the whether they produce nodularin or not.

Materials and methods

Culture conditions and experimental design

The experimental design contained a multiple matrix approach with different factorial designs for the Cyanobacteria tested, using a combination of different atmospheric CO_2 treatments of high CO_2 (HC), low CO_2 (LC), culture medium containing nitrogen in the form of NaNO_3 or not (N+ and N-) and the ability to produce the hepatotoxin, nodularin (+ and -) (Fig. 1a).

In total, six species of heterocystous filamentous Cyanobacteria belonging to two families, *Nostocaceae* and *Aphanizomnaceae*, within the order Nostocales, were selected for investigation. Four representative species of the genus *Nostoc* were analysed, with two being nodularin producers,

ie *Nostoc punctiforme* sp. 73.1 and *Nostoc muscorum* sp. 65.1 and two non-nodularin producing species: *Nostoc punctiforme* sp. 40.5 and *Nostoc entophyllum* sp. C1.8 (Gehring et al. 2010, 2012). Two representative species of the genus *Nodularia*, were investigated, with one nodularin producing species, *Nodularia spumigena* CCY9414 (Voss et al. 2013) and its related non-toxic mutant strain *Nodularia spumigena* NSBL06 [analogous to *N. spumigena* NSBL05 (Bolch et al. 1999; Moffit et al. 2001)] and the non-toxic benthic species of *Nodularia harveyana* SAG 44.85 (Lyra et al. 2005; Řeháková et al. 2014). The combination of the three variable factors, atmospheric CO_2 levels, nitrogen content and toxin production, generated four different factorial designs for the species tested (Fig. 1b).

The terrestrially isolated *Nostoc* species were maintained since their isolation on the nitrogen free medium, BG11₀, medium with ferric ammonium citrate replaced with ferric citrate (Gehring et al. 2010), thereby ensuring their ability to fix nitrogen was not lost. Three months prior to this study, the *Nostoc* species were also subcultured into BG11 medium containing NaNO_3 (17.6 mM). The aquatic diazotrophic species *Nodularia spumigena* CCY9414 (Culture Collection Yerseke), *Nodularia spumigena* NSBL 06 (kindly provided by Hanna Mazur-Marzec, University of Gdansk) and the benthic *Nodularia harveyana* SAG 44.85 (Culture Collection of Algae, SAG, Georg August University, Göttingen) were cultivated in nitrogen free brackish sea water medium (F/2) with a salinity of 10 containing vitamins (UTEX, Austin). The benthic species, *Nodularia harveyana* SAG 44.85, required the addition of 5 ml l⁻¹ of soil extract.

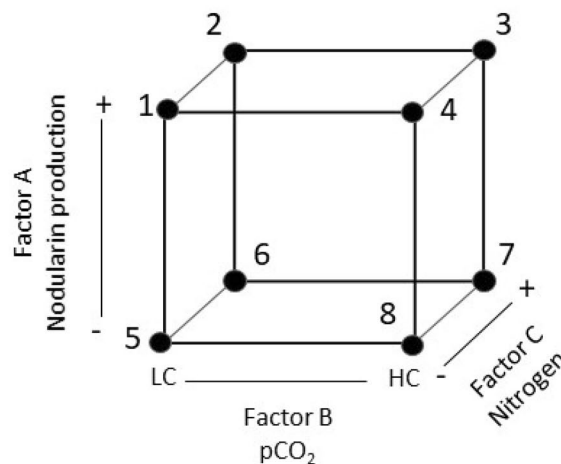
Fifty ml of stationary phase cultures were inoculated into 150 ml of the appropriate media in a Fernbach flask (Duran, d=45 mm) for maximal volume to surface area ratio, and placed at the control or experimental conditions for 14 days to allow them to adjust to their new conditions (Eichner et al. 2014). The inoculum cultures were then diluted 1:1 with fresh medium and divided into two ventilated T₁₇₅ polystyrene cell culture flasks (Greiner). In this manner three toxin producing species (n=3), namely *Nodularia spumigena* CCY9414, *Nostoc punctiforme* sp. 73.1 and *Nostoc muscorum* sp. 65.1 and four control, non-toxin producing species (n=4), namely *Nodularia spumigena* NSBL06, *Nodularia harveyana* SAG 44.85, *Nostoc punctiforme* sp. 40.5 and *Nostoc entophyllum* sp. C1.8, were studied (Fig. 1). The flasks were laid flat to minimise shading effects, resulting in a culture depth of 1 cm that maximised gas exchange at the culture surface (Herrmann and Gehring 2019). Experimental cultures were exposed to elevated CO_2 of 2000 ppm (defined here as “High CO_2 ”—HC), 10:14 h light:dark cycle, 22 °C, 60% humidity and 130 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Plant growth chamber E-22L, Percival, USA). Control cultures were exposed to CO_2 at present day level, ~ 440 ppm in Kaiserslautern, Germany (defined here as “Low CO_2 ”—LC),

Fig. 1 Species characteristics and three factorial design chosen for the seven *Nostocaceae* species tested (a). Factors include the ability to produce nodularin (Factor A); the CO₂ treatment (Factor B) with present day levels (440 ppm—“Low CO₂” LC) and elevated CO₂ (2000 ppm- “High CO₂”-HC), and nitrogen availability (Factor C) with cultures grown either diazotrophically or in N-replete medium (N+an N-). The different factorial designs in a) result from the combination of factors A–C for the different species illustrated in (b)

(a) Species and experimental characteristics

Species	N ₂ fixation rate assessed	Toxin producer	Factorial design see b)	
			Growth and physiology	Nodularin analysis
<i>Nostoc punctiforme</i> sp. 73.1	✓	✓	1,2,3,4	1,2,3,4
<i>Nostoc punctiforme</i> sp. 40.5	-	-	6,7	/
<i>Nostoc muscorum</i> sp. 65.1	-	✓	2,3	1,2,3,4
<i>Nostoc entophyllum</i> sp. C1.8	-	-	6,7	/
<i>Nodularia spumigena</i> CCY9414				
	✓	✓	1,4	1,2,3,4
<i>Nodularia spumigena</i> NSBL206				
	✓	-	5,8	/
<i>Nodularia harveyana</i> SAG 44.85				
	✓	-	5,8	/

(b) Three- Factorial design



No.	A Nod	B pCO ₂	C N
1	+	LC	-
2	+	LC	+
3	+	HC	+
4	+	HC	-
5	-	LC	-
6	-	LC	+
7	-	HC	+
8	-	HC	-

with the same culture conditions as above. Different parameters were sampled on days 7- and 14-post inoculation. The numbers of replicates for each sampling day and total numbers are provided in Table 1. In our probe study, a trade off was made between using a variety of species (toxic and non-toxic) from aquatic and terrestrial origin versus increasing the number of replicated incubation bottles per sampling time point. We chose to use a repeated measure approach for most of the parameters with two replicate incubation bottles and two sampling time points. In the case of N₂ fixation, we ended up with technical replicated sampling from each bottle at one sampling time point, applying pseudo-replication in this case.

Determination of growth curves based on optical density were set up separately from the experimental bottles after the adjustment phase with a start inoculum of OD₆₅₀ of ~0.1 and 200 µl pipetted into 6 wells of a 96 well microtiter plate for each Cyanobacterial culture. Individual Cyanobacteria were grown in their appropriate medium, both diazotrophically and in nitrogen replete medium, at LC and HC. Cultures were resuspended by pipetting and shaking just before the OD₆₅₀ was read in a Multiscan Microtitre plate reader (Thermo Scientific) over 14 days post inoculation at experimental conditions identical to the cell culture flasks. To ensure growth rates based on optical density measurements in the 96 well microtiter plate were not biased compared to

Table 1 Number of replicate incubation bottles and sampling per day for the seven species tested for the LC (440 ppm) and HC (2000 ppm) treatment. 2+2 indicates technical replicates from 2 incubation bottles at one time point

Investigated Parameter	Treatment	Sampling day		No. of replicates per sampling day		Total no. of replicates
		7	14	7	14	
CO ₂ uptake	LC	●	●	2	2	4
	HC	●	●	2	2	4
Dark respiration	LC	●	●	2	2	4
	HC	●	●	2	2	4
Chlorophyll a	LC	●	●	2	2	4
	HC	●	●	2	2	4
Nodularin	LC		●		3	3
	HC		●		3	3
PON/POC	LC		●		2+2	4
	HC		●		2+2	4
N ₂ fixation	LC	<i>Nostoc sp.</i> *	●		2	2
		<i>Nodularia sp.</i>	●		2+2	4
	HC	<i>Nostoc sp.</i> *	●		2	2
		<i>Nodularia sp.</i>	●		2+2	4
Incubation and sampling in 96 well- microtiter plates						
Growth based on OD ₆₅₀ **	LC					
	HC					
Read on days 3 – 7 and 10, 11, 12 and 14, 6 replicates each						
Incubation and sampling in cell culture flasks						
Growth based Chl a.	LC					
	HC					
Sampling at day 3, 5, 7, 10, 12, 14 3 replicates each						

*Determined only for *Nostoc punctiforme* sp. 73.1 (–N)

**Readings were repeated on days listed on cultures resuspended in 96 well- microtiter plates every over a period of 14 days. Calculation was done for the period of day 3–14 (10 absorbance readings)

the actual experimental set-up in culture flasks, we reputed growth rates determination in culture flasks for 14 days of incubation. Growth curves were therefore set up in culture flasks, in triplicate for terrestrial *Nostoc* species *Nostoc punctiforme* sp. 73.1, *Nostoc muscorum* sp. 65.1, *Nostoc punctiforme* sp. 40.5 and *Nostoc entophyllum* sp. C1.8 in freshwater growth medium under both diazotrophic (N–) and non—diazotrophic (N+) conditions at LC and HC

atmospheric conditions. Similarly, growth curves were established in triplicate for *Nodularia spumigena* CCY9414 and *Nodularia spumigena* NSBL06 in brackish sea water growth medium, in both N-replete and N-free medium. T₇₅ ventilated suspension culture flasks (Sarstedt, Germany) containing 75 ml of the appropriate medium, were inoculated with stationary phase cultures from the respective atmospheres to give a starting Chl *a* content of 0.1 µg ml^{–1}.

Two ml samples were collected from agitated cultures on day 3, 5, 7, 10, 12 and 14 for Chl. *a* determinations (below). Two ml of culture material was harvested for nodularin analysis on day 14 (below) and a 20 ml volume was centrifuged, drained and dried in a 60 °C oven to obtain the biomass per volume.

Carbonate chemistry

The pH was determined on day 14 from sample filtrates using an electrode (Radiometer analytical PHM210, France) calibrated with a three-point calibration using NBS (National Bureau of Standards) buffers giving values of pH relative to the NBS scale. Total alkalinity (A_T) was determined 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 $\pm 2 \mu\text{mol kg}^{-1}$. Total carbon (C_T) and $p\text{CO}_2$ in the growth media were calculated using CO₂SYST (Lewis et al. 1998). Media control carbonate chemistry was similarly assessed with experimentally obtained values for pH and total alkalinity (TA) determined by manual titration (Dickson et al. 2007) and calculated using the Seacarb package in RStudio version 1.0.153, as input data (Herrmann and Gehringer 2019).

Net CO₂ uptake/net photosynthesis (NP)

Culture material was removed by pipetting under sterile conditions in a clean bench in LC and HC conditions on days 7 and 14 representing mid to late exponential growth phases. Harvested Cyanobacteria were filtered onto a 3 μm SSWP (Millipore) glass fibre filter (Ritchie 2008), placed onto an appropriate, moist agar plate and incubated under experimental conditions until CO₂ uptake determinations (between 1 and 4 h after sampling) by means of CO₂ gas exchange measurements (GFS 3000, WALZ, Effeltrich, Germany). Bacterial covered filters of LC acclimated cultures were placed in the sample cuvette and CO₂ uptake determined at 80% humidity at 440 ppm (Herrmann and Gehringer, 2019), while for HC acclimated cultures measurements was done at 1500 ppm CO₂ (readings at 2000 ppm were too unstable at 80% humidity in the sample cuvette of the GFS 3000). The respiration rate was determined for each filter after 5 min dark incubation at the start and end of the measuring period to ensure the cultures were not stressed. CO₂ assimilation rates were determined at 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (approximate light saturation point for all Cyanobacteria used in this study, determined from light curves), and expressed per μg chlorophyll *a*. Net photosynthesis (NP) rates, representing the total assimilation of CO₂ minus the CO₂ released during respiration, were calculated.

Chlorophyll a determination

Chlorophyll *a* was extracted from the bacterial filter discs used for the gas exchange experiments above. After CO₂ uptake measurements, each filter was placed in a 2 ml centrifuge tube containing 100 mg of 0.1 mm zirconia silica beads (BioSpec) to which 1.5 ml 90% HPLC grade methanol was added (Meeks and Castenholz 1971). The samples were bead-beated (Retch, Germany) for 1 min at 30 beats per min and incubated at 4 °C in the dark overnight. Samples were subsequently centrifuged at 10 000 rcf for 5 min at 20 °C and the OD₆₆₅ was determined (Lambda 35 UV/VIS spectrometer, Perkin-Elmer). Chlorophyll *a* content was calculated using the equation: $\text{Chl } a \mu\text{g ml}^{-1} = \text{OD}_{665} \times 12.7$ (Meeks and Castenholz 1971). Cell pellets obtained from centrifuging two ml of culture were extracted in the same manner to generate the Chl *a* based growth rates.

Nodularin analysis

Samples for toxin determinations were obtained on day 14 of the growth curves for nodularin producing Cyanobacterial cultures. A 2 ml volume of culture material was centrifuged and the cell pellet drained. One hundred mg of 0.1 mm zirconia silica beads (BioSpec) were added to the pellet with 1.5 ml 70% HPLC grade methanol (Gehringer et al., 2012). The samples were lysed by bead beating as above and incubated at room temperature in the dark overnight. The following morning the samples were vortexed, the lysed cell material removed by centrifugation as above, and the supernatant fluid used in a competitive ELISA assay (Abraxis #522015, Eurofins, Luxembourg) following the manufacturer's instructions. The amount of toxin extracted for each nodularin producing Cyanobacterium under diazotrophic and non-diazotrophic conditions was calculated from the standard curve ($R^2 = 0.9937$) and expressed as total soluble cellular nodularin content per dry biomass [$\text{ng nodularin. } \mu\text{g dry weight}^{-1}$].

Particulate organic matter and N₂ fixation

The PON and POC content were measured for *Nodularia* cultures and *Nostoc punctiforme* sp. 73.1 grown in N-free medium on day 14. Due to budget constraints, the remaining *Nostoc* cultures were not studied. Filters containing culture samples were trimmed, sectioned, then loaded into tin capsules and palletised for isotopic analysis. Measurement was done by means of flash combustion in a Carlo Erba EA 1108 at 1020 °C in a Thermo Finnigan Delta S mass-spectrometer. Calibration material for N and C analysis was acetanilide (Merck). N₂ fixation activity was determined by incubating cultures in two replicates per treatment with bubble addition of ¹⁵N–N₂ enriched gas (99% ¹⁵N₂) for 24 h, guaranteeing

sufficient dissolution of the ^{15}N gas in the incubation bottle (Wannicke et al. 2018a). Tracer incubations were terminated by gentle vacuum filtration (<200 mbar) of the culture material through pre-combusted GF/F filters (Whatman) that were then dried at 60 °C, analysed and the N_2 fixation rates calculated (Montoya et al. 1996). Two technical replicates were conducted per bottle.

Statistical analysis

Statistical analyses were done either by using the Student's *t* test or Mann–Whitney Rank Sum Test comparing the mean effect sizes and mean values or by using one-way and repeated measures ANOVA to determine the CO_2 treatment effect. It has to be noted that in the case of *Nostoc punctiforme* sp. 73.1, only two samples for N_2 fixation were successfully measured. Two replicates were lost due to an autosampler error during processing. No statistical analysis comparing the treatment groups was applied in this case.

Prior to statistical analysis, data were tested for normality and homogeneity of variances using Wilk-Shapiro and Levene's tests. All analyses were performed using the software SigmaPlot 13 (Systat Software Inc., San Jose, CA, USA).

Response ratios and weighted mean effect sizes

To investigate a possible modulating effect of toxin production in response to elevated CO_2 and of elevated CO_2 on volume and dry weight specific nodularin production, we determined the response ratio, i.e. $\ln\text{RR} = \left(\frac{\bar{x}_T}{\bar{x}_C}\right)$ for net photosynthesis, dark respiration, growth and N_2 fixation in selected Cyanobacteria. Here $\ln\text{RR}$ is the natural-log proportional change in the means (\bar{x}) of the CO_2 treatment (T, i.e. HC) and control group (C, i.e. LC). Negative values of $\ln\text{RR}$ denote lower rates/ growth at elevated CO_2 compared to control, and vice versa.

To examine the modulating effect of nodularin production over all species tested, pooled $\ln\text{RR}$ values were combined to give a mean effect size (i.e. Cohen's *d*). A weight was assigned to each $\ln\text{RR}$ obtained from individual species which was inversely proportional to its sampling variance (DerSimonian and Laird 1986) as represented by the following equation: $d = \frac{\bar{x}_T - \bar{x}_C}{\text{SampleSD}_{\text{pooled}}}$. Sub-group calculations were done for the groups "nodularin producer" and "non-nodularin producer" (see Fig. 1 for the toxin status of each Cyanobacterium investigated) and for the nodularin production per volume and per dry weight. To calculate the weighted mean effect sizes, their significance and 95% confidence intervals, a random effect model was applied (DL = DerSimonian–Laird estimator) using Meta-Essential (Suurmond et al. 2017a, b).

Results

Carbonate chemistry

Carbonate chemistry of the media confirmed that experimental application of a continuous atmospheric gas supply ensured enrichment with CO_2 in cultures grown at elevated CO_2 levels. The $p\text{CO}_2$ in the growth media of cultures incubated at HC in the *Nostoc* cultures was determined to be $1987 \pm 42 \mu\text{atm}$ for N-free media, while the $p\text{CO}_2$ in the LC treatment was $293 \pm 38 \mu\text{atm}$. *Nodularia* cultures displayed a mean $p\text{CO}_2$ of $1701 \pm 83 \mu\text{atm}$ in N-replete brackish seawater medium (Suppl. Table 1). Control cultures at LC conditions revealed significantly reduced $p\text{CO}_2$ availability at $232 \pm 26 \mu\text{atm}$. Control medium at HC was $2728 \pm 323 \mu\text{atm}$ and $2203 \pm 405 \mu\text{atm}$ for fresh and brackish N-free media respectively. Also, carbonate chemistry determined in the experimental bottles showed significant differences between LC and HC treatments (Suppl. Table 1). The N-free media TA values determined agreed with previously published data (Wannicke et al. 2012) in low nutrient media. The TA values for experimental cultures grown at HC in media containing N were also exceedingly high, suggesting interference of biologically synthesised compounds interfering with the TA assessment. These values were therefore not reported.

Effect of elevated CO_2 on Cyanobacterial growth

The growth curves for each species grown under LC and HC conditions highlight the different responses between Cyanobacterial cultures (Suppl. Fig. 1, 2, 3) to atmospheric CO_2 and / or nitrogen availability. The data for *Nostoc* species 65.1, 40.5 and C1.8, under N limitation, are not presented as the NP readings fell below the level of detection in the gas exchange measurements.

Nostoc punctiforme sp. 73.1 exhibited higher growth rates at HC conditions than at LC, with higher growth rates observed under N replete conditions. *Nostoc punctiforme* sp. 40.5 did not show a significant response towards HC. On the other hand, both *Nostoc muscorum* sp. 65.1 and *Nostoc entophyllum* sp. C1.8 displayed lower growth rates at HC (Suppl. Table 2).

Nodularia harveyana SAG 44.85 and *Nodularia spumigena* CCY9414 showed increased growth at HC compared to LC grown cultures for the time interval 0–14 days (Suppl. Table 3), while *Nodularia spumigena* NSBL206 displayed lower growth rates at HC.

Growth rates determined in 96-well microtitre plates and culture flasks are mostly in agreement (Suppl. Fig. 4). In *Nostoc* species, growth rates calculated from optical

density in the 96-well microtiter plate were mostly lower than those derived from tracking Chl a content in the culture flasks, with the largest deviation of ~35% in *Nostoc entophyllum* sp. C1.8 and *Nostoc punctiforme* sp. 73.1 grown at N-replete conditions, at LC and HC respectively. In *Nodularia* species, the largest deviations in recorded growth rates were 28% for *N. spumigena* NSBL206 and 17% for *N. spumigena* CCY9414, grown diazotrophically, at LC and HC respectively, with higher growth rates determined when optical density was used to track growth.

The endpoint biomass dry weight (µg/ml) indicates a general trend of increasing biomass under HC conditions compared to LC growth controls (Supp. Table 1). *Nostoc* species increased their biomass significantly under HC conditions under both diazotrophic (170.7 ± 52.2 µg ml⁻¹) and N-replete (288.0 ± 94.6 µg ml⁻¹) conditions, while *Nodularia* species showed a non-significant trend towards elevated biomass under HC conditions under both diazotrophic (534.0 ± 343.0 µg ml⁻¹) and N-replete (622.0 ± 333.7 µg ml⁻¹) conditions.

Effect of elevated CO₂ on photosynthesis

A statistically significant increase in net photosynthesis was observed in all species tested at HC compared to the LC control cultures (Fig. 2), except for *Nodularia harveyana* SAG 44.85 which displayed a significant reduction (p ≤ 0.005) in photosynthesis at HC. This effect was independent of inorganic nitrogen in the growth media being present or absent in *Nostoc punctiforme* sp. 73.1. Overall, photosynthesis rates pooled for all *Nostoc* species over all treatments was significantly higher than pooled for *Nodularia* species (F = 36.3, n = 92/22, p ≤ 0.001). There was a trend towards elevated dark respiration at HC in *Nostoc punctiforme* sp. 73.1 and *Nodularia harveyana* SAG 44.85 grown diazotrophically (Suppl. Fig. 5). For *Nodularia spumigena* NSBL206 this trend was statistically significant (Suppl. Fig. 5).

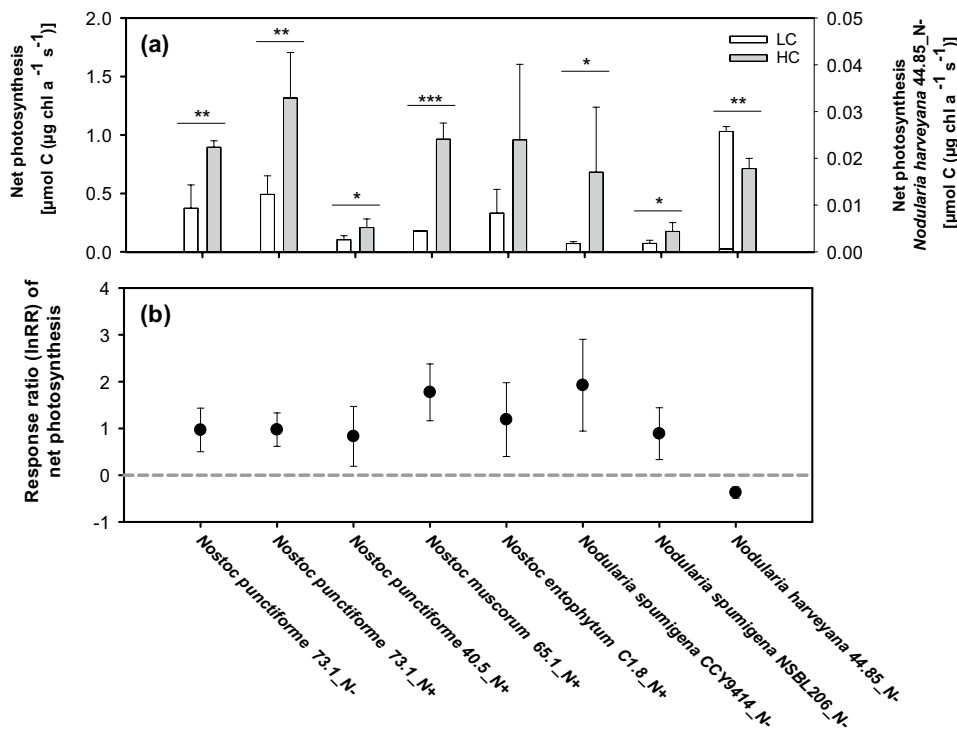


Fig. 2 **a** Net photosynthesis (NP) in control cultures (LC-440 ppm) and cultures grown at elevated CO₂ (HC-2000 ppm). Bars represent mean and standard deviation of four measurements (day 7 + day 14). Second y-axis (RHS) refers to *Nodularia harveyana* SAG 44.85. Significant differences between measurements are indicated by *p ≤ 0.05, ** p ≤ 0.005, ***p ≤ 0.001 according to repeated -measure ANOVA and **b** Response ratio of net photosynthesis was calculated by deter-

mining the natural logarithm of the ratio of the NP rate of HC cultures by those of cultures grown at LC (lnRR). Data is presented as means and 95% confidence interval (CI). The horizontal grey line indicates lack of response to the CO₂ treatment (i.e. RR = 0). If the CI crossed the 0 response line, the effect of elevated CO₂ is considered as non-significant. Mean and CI > 0 indicate a stimulation by elevated CO₂. Mean and CI < 0 indicate a negative effect of elevated CO₂

Effect of elevated atmospheric CO₂ on nodularin production per culture volume

Comparing dry weight volume specific biomass nodularin content revealed a statistically significant impact of HC growth conditions in two of the three toxin producing species, *Nostoc punctiforme* sp. 73.1 and *Nodularia spumigena* CCY9414. A significant decrease in nodularin content per dry weight was observed at elevated CO₂ in *Nostoc punctiforme* sp. 73.1 when grown diazotrophically, while there was no significant impact in nitrogen containing growth media (Fig. 3a). No significant impact of HC on nodularin content was detectable in *Nostoc muscorum* sp. 65.1, regardless of N availability (Fig. 3b). A significant decrease in dry weight specific nodularin content was apparent for *Nodularia spumigena* CCY9414 when grown under diazotrophic or N-replete conditions (Fig. 3c) at HC.

The effect of elevated CO₂ on nodularin production was strongly influenced by the normalisation of actual toxin concentrations. When comparing volume specific nodularin concentration (ng nodularin per culture volume analysed) a strong increase in nodularin production was observed at elevated CO₂ for *Nostoc punctiforme* sp. 73.1 under N-replete conditions (N+) when compared to diazotrophically grown *Nostoc punctiforme* sp. 73.1, while there was only a slight increase for *Nodularia spumigena* CCY9414 (Suppl. Fig. 6) cultured diazotrophically. Dry weight specific nodularin content was decreased in diazotrophically grown *Nostoc punctiforme* sp. 73.1 in contrast to the decrease observed in nodularin levels of *Nodularia spumigena* CCY9414 grown under both nutrient conditions (Fig. 3).

Effect on N₂ fixation

N₂ fixation rates in both *Nodularia* species were significantly elevated by a factor of 6 for biomass specific rates ($t=3.85$, $p=0.001$, $n=24$ for *Nodularia*/ $n=4$ for *Nostoc*) and 17 for volume specific rates ($t=2.83$, $p=0.008$, $n=24$ for *Nodularia*/ $n=4$ for *Nostoc*, Fig. 4) when compared to rates determined for *Nostoc punctiforme* sp. 73.1. These rates should be interpreted carefully, especially for *Nostoc punctiforme* sp. 73.1, due to the low number of replicates ($n=2$). Moreover, volume specific N₂ fixation rates were significantly higher for *Nodularia spumigena* CCY9414 and *Nodularia harveyana* SAG 44.85 under HC growth conditions when compared to the LC cultures ($F=34.9$, $p=0.01$, $n=4$ and $F=11.7$, $p=0.04$, $n=4$), respectively (Fig. 4a). There was no trend in N₂ fixation in *Nodularia spumigena* NSBL206.

N₂ fixation rates normalized to particulate organic nitrogen were significantly decreased in the two non-toxin producing *Nodularia* species, *Nodularia harveyana* SAG 44.85 and *Nodularia spumigena* NSBL206 at elevated pCO₂ (HC)

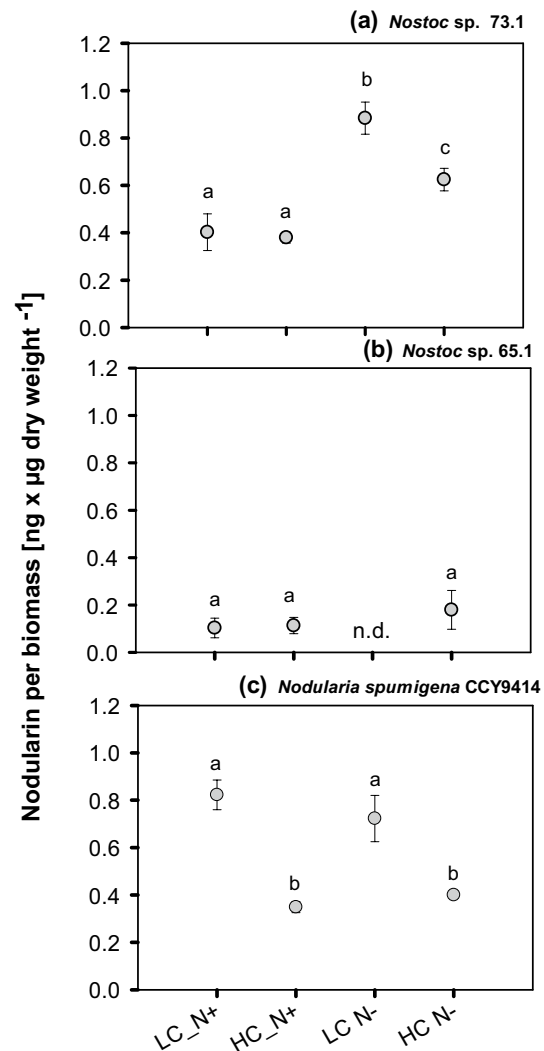


Fig. 3 Total soluble cellular nodularin content per µg dry weight for control cultures (LC-440 ppm) and cultures grown at elevated CO₂ (HC-2000 ppm). Values represent mean and standard deviation determined after 14 days of incubation ($n=3$). Significant differences are indicated by different letters to the level of $p \leq 0.05$ according to one-way ANOVA

(Fig. 4a). This may reflect the significant increase ($p \leq 0.05$) in PON levels recorded for all three *Nodularia* cultures under HC conditions, which were elevated by a factor of 3–6.5 under HC conditions (Suppl. Fig. 7a). The BNF rates determined for the *Nostoc punctiforme* sp. 73.1 ($n=2$) showed no definitive trend at HC growth conditions as seen for *Nodularia spumigena* CCY9414 and *Nodularia harveyana* SAG 44.85, at HC.

Modulation of CO₂ response in nodularin and non-nodularin producer

Weighted mean effect sizes for the subgroups, nodularin producer and non-nodularin producer, were positive for NP

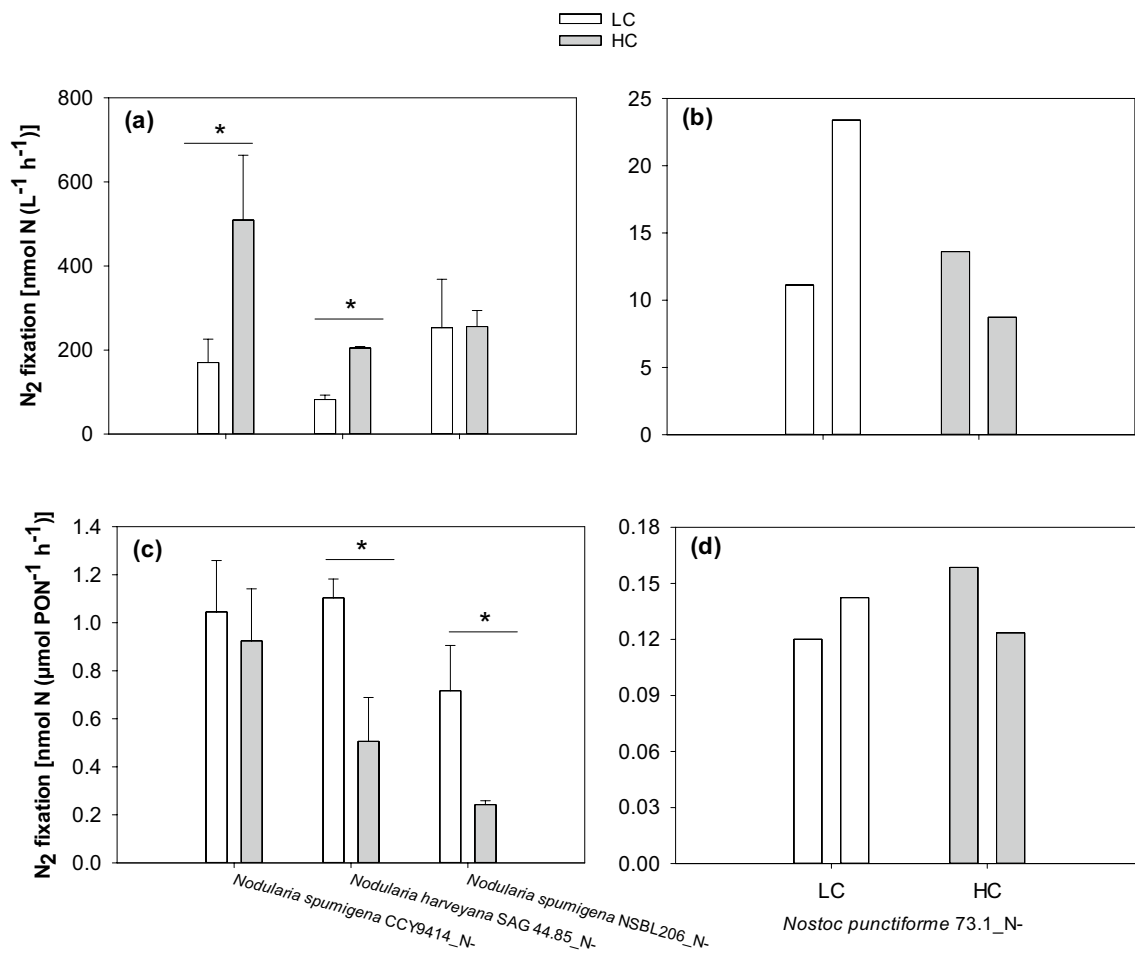


Fig. 4 Volume specific and biomass specific N₂ fixation rates determined for *Nodularia* sp. (**a**, **c**) and *Nostoc punctiforme* sp. 73.1 (**b**, **d**) grown at LC (440 ppm) and HC (2000 ppm). Bars in **a** and **c** represent mean and standard deviation of four replicates, bars in **b** and

d represent single measurements. Significant differences between treatments are indicated by * $p \leq 0.05$ according to repeated -measure ANOVA

indicating a positive effect of the high CO₂ treatment on net photosynthesis. Nodularin producing Cyanobacteria displayed a significantly higher positive response towards HC compared to non-nodularin producers ($p \leq 0.006$, Student's *t* test, Fig. 5). Weighted mean effect sizes of dark respiration differed in the two subgroups. Nodularin producers showed no significant effect of HC for dark respiration (overlap of confidence interval with 0-response line, Fig. 5), while non-nodularin producer displayed a positive mean effect size. Weighted mean effect sizes of growth rates showed a significant difference in-between the two subgroups. Mean effect size of non-nodularin producing Cyanobacteria did not deviate from the 0-response line indicating no effect of CO₂ treatment on growth, while the nodularin producers displayed a significant positive response to HC. Contrasting patterns were visible for N₂ fixation, depending on the mode of normalisation of rates, namely volume or PON. Weighted mean effect sizes of biomass specific N₂ fixation

showed opposing directions and a significant difference in the subgroups ($p \leq 0.001$, Student's *t* test) with a significant decrease in N₂ fixation at HC in the non-nodularin producer and a no effect on nodularin producer (overlap of confidence interval with 0-response line, Fig. 5). Mean effect sizes of volume specific N₂ fixation rates showed an opposing trend. Non-nodularin producers displayed significantly elevated BNF rates at HC, while nodularin producers displayed a non-significant increase at HC.

Discussion

The recent report by the Intergovernmental Panel on Climate Change indicates that CO₂ emission rates are not being reduced as rapidly as desired, suggesting levels of CO₂ which will most likely exceed 1000 ppm by the year 2100 for the worst-case-scenario (RCP8.5) (IPCC 2019).

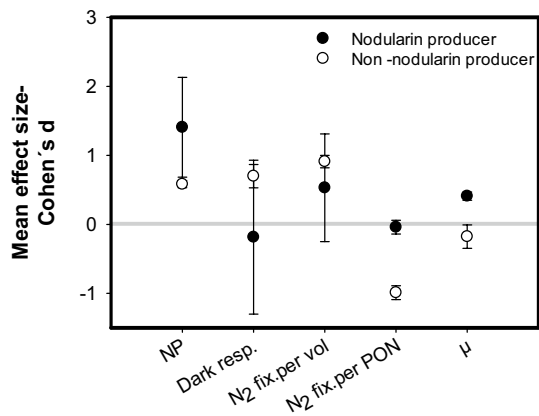


Fig. 5 Weighted mean effect sizes (Cohen's *d*) of net photosynthesis (NP), respiration, growth and N₂ fixation for the two subgroups "Nodularin producer" (black circles, for details on Cyanobacteria see Fig. 1a) and "Non-nodularin producer" (white circles, for details on Cyanobacteria see Fig. 1a). Data is presented as means and 95% confidence interval (CI). The horizontal grey line indicates lack of response to the CO₂ treatment (i.e. mean effect size=0). If the CI crossed the 0 response line, the effect of elevated CO₂ is considered as non-significant. Mean and CI > 0 indicate a stimulation by elevated CO₂. Mean and CI < 0 indicate a negative effect of elevated CO₂

Recent reviews have summarised the literature regarding the response of marine and freshwater bloom forming Cyanobacteria to elevated CO₂ levels (Huisman et al. 2018; Visser et al. 2016; Wannicke et al. 2018b), suggesting an increase in bloom formation, possibly favouring diazotrophs, as they would be less susceptible to N-limitation (Gehring and Wannicke 2014). While numerous studies have been conducted on toxin and non-toxin producing *Microcystis aeruginosa* strains under elevated CO₂ conditions, little data has been accumulated as to the effects on diazotrophic Cyanobacteria, especially terrestrial *Nostoc* species (Gehring and Wannicke 2014). In this study, the effects of elevated atmospheric CO₂ on various growth parameters of six species of diazotrophic, heterocystous Cyanobacteria of two families of the order Nostocales were assessed. The weighted mean effect sizes (Cohen's *d*) of three nodularin producing Cyanobacteria were compared to four non-nodularin synthesising Cyanobacteria with respect to NP, growth and BNF rates (Fig. 5). Nodularin producers tended to have higher NP rates under HC conditions than non-toxin producers, accompanied by increased growth rates. Non-toxic Cyanobacteria in contrast showed increased respiration at HC conditions, suggesting increased respiratory or oxidative stress. This effect was largely driven by the negative response of *Nostoc punctiforme* sp. 73.1. Due to the lack of individual species replication, these investigations need to be repeated to make statistically robust conclusions. The BNF rates of both nodularin producers and non-toxic species were unaffected by HC growth conditions when normalised

against PON given that the 95% confidence interval crosses the zero line in the Cohen's *d* plot (Fig. 5), while toxin producers had slightly lower rates. This trend appeared inverted if BNF were normalised against culture volume, where non-nodularin producers were negatively affected by HC, while nodularin producers showed no effect. These trends suggested that nodularin producing diazotrophs did indeed respond differently to changing elevated CO₂ levels compared to non-nodularin producers. We then proceeded to investigate these results in more detail at the individual genus, and species level.

Response of elevated CO₂ on NP and growth

All aquatic and terrestrial diazotrophic Cyanobacteria investigated in this study fixed atmospheric CO₂ in the range of 0.1–1.5 μmol C. ng Chl *a*⁻¹ s⁻¹, whether under N-replete or diazotrophic conditions. Additionally, our study has confirmed that most *Nostoc* species and *Nodularia spumigena*, grown at 2000 ppm CO₂, have the capacity for significantly higher NP at HC conditions (Fig. 2), indicating that they are not functioning at saturation under current atmospheric levels of CO₂. The benthic species, *N. harveyana* SAG 44.85, however showed a significant reduction in NP with HC. A similarity search conducted using BLASTn (Altschul et al. 1990) on the genome of *Nodularia spumigena* CCY9414, found that this strain carries a gene (NSP_RS09630) with 75% identity to the *BicA* gene of *Microcystis aeruginosa* PCC7806, thereby suggesting that it can benefit from increased HCO₃⁻ in the media and thus, increase its NP rates accordingly (Visser et al. 2016). Gas exchange measurements were used to assess NP (Herrmann and Gehring 2019) as most Cyanobacteria are known to encode the CO₂ converting enzymes, NDH-I₄ and NDH-I₃ (Visser et al. 2016), necessary for the direct, non-energy demanding conversion of CO₂ to bicarbonate for transport to the carboxysome. As non-sequenced environmental isolates were used, there was no information regarding the status of bicarbonate transporters of all the Cyanobacteria under investigation.

Alkalinisation of the culture medium of *Nostoc* species grown under N-replete conditions at both HC and LC culture conditions was recorded, whereas alkalinisation was only seen for *Nodularia* grown under N-replete conditions at LC conditions (Supp. Table 1). The changes in pH do not follow the observed changes in biomass, suggesting species specific responses to changes in pH and dissolved inorganic carbon availability.

In our study, a CO₂ enriched atmosphere led to an increase in inorganic carbon in the control media flasks, while alkalinity was kept constant and pH decreased. Often discussed is the effect of reduced pH by adding acid to keep inorganic carbon stable while total alkalinity and pH decrease. For example, a study by Berge et al. (2010)

showed that phytoplankton of the genera dinoflagellates, cryptophytes, diatom and prymnesiophyte were resistant in terms reduced pH and did not increase or decrease their growth rates according to ecological relevant ranges of pH from 7.0 to 9.0. More recently, the response of *Raphidiopsis raciborskii* to changes in pH and inorganic carbon in water was assessed (Vilar and Molica 2020). The growth of the Cyanobacterium, *R. raciborskii* was increased with the addition of sodium carbonate and air bubbling, however, saxitoxin production was reduced. Additionally, the authors observed that pH changes were related to significant changes in cellular saxitoxin levels. In general, the potential effect of pH changes on neither the growth, nor nodularin production, of the Nostocales strains investigated in our study, have been characterised and published in the past.

Especially the Baltic Sea is depleted with CO₂ during summer time due to the high draw down of dense phytoplankton blooms with high photosynthetic activity (e.g. Huisman et al. 2018), as well as poor diffusion of CO₂ in water and the slow equilibrium between CO₂ and HCO₃⁻ (e.g. Ibelings and Maberly 1998). Photosynthesis in biocrusts is also often limited by CO₂ availability, especially when flooded or desiccated (Tuba et al. 1998; Jauhiainen and Silvola 1999; Lange 2002; Botting and Fredeen 2006; Toet et al. 2006).

The reduction in NP seen for the benthic *Nodularia harveyana* SAG 44.85, suggests that it may tightly regulate its Ci uptake mechanisms, as elevated CO₂ levels of up to 3000 µatm can occur in benthic layers due to high organic matter decomposition and remineralisation (Haynert et al. 2012). The capacity to tightly regulate C assimilation is an important prerequisite for reducing respiratory stress in this environment. The increase in dark respiration in *N. harveyana* SAG44.85 suggests it may indeed be under respiratory stress at HC conditions.

Nodularin production under elevated atmospheric CO₂ exposure

Intracellular nodularin content showed significant variation dependent on the Cyanobacterium and/or atmospheric CO₂ content (Fig. 3). *Nostoc* sp. 65.1 appears to constitutively produce nodularin at low levels, independent of medium N content and atmospheric CO₂ levels. This *Nostoc* species also exhibited the lowest growth rate overall (Supplementary Table 2), which prevented the generation of sufficient biological biomass under diazotrophic growth conditions for NP and BNF determinations. The nodularin content of *Nostoc* sp. 73.1 was significantly raised under diazotrophic growth conditions at both atmospheres. NP rates (Fig. 2) and growth rates (Supplementary Table 2) of *Nostoc* sp. 73.1 exceeded those of *Nostoc* sp. 65.1 under N-replete conditions, factors

that may contribute to its higher levels of intracellular toxin production under diazotrophic conditions.

Nodularia spumigena CCY 9414 exhibited a very different nodularin synthesis profile, with cultures grown at LC conditions containing significantly more nodularin per dry weight than the cultures in HC conditions, irrespective of medium N content (Fig. 3). The high NP rates observed under HC conditions (Fig. 2), combined with the increased growth rates at HC under diazotrophic conditions suggest that the cells were N depleted and thus not expending resources to produce nodularin. However, the particulate organic nitrogen levels of the HC grown cultures (Supplementary Fig. 7) suggest that the cells were not N-depleted and that some other regulatory mechanism was suppressing nodularin synthesis, compared to the LC cultures.

Biological nitrogen fixation

All *Nodularia* investigated exhibited significant increases in their culture volume PON content under HC culture conditions (Supplementary Fig. 7). However, increased BNF rates per volume were only significantly raised ($p \leq 0.05$) for *Nodularia spumigena* CCY 9414 and *Nodularia harveyana* SAG 44.85 under HC, while those for *N. spumigena* NSBL06 remained unaffected. If the BNF rates are expressed per PON content, the LC exposed cultures of *Nodularia harveyana* SAG 44.85 and *N. spumigena* NSBL06 are significantly higher ($p \leq 0.05$) than HC grown cultures (Fig. 4), suggesting inhibition of BNF under HC conditions. A recent study (Boatman et al. 2019) found that dark respiration rates were up to 5 times higher in *Trichodesmium erythraeum* IMS101 cultures exposed to elevated levels of CO₂ (720 µmol mol⁻¹). While increases in dark respiration were observed for the non-toxin producing *Nodularia spumigena* NSBL06 and *Nodularia harveyana* SAG 44.85 (Supplementary Fig. 5), no increase in BNF rates per PON were recorded (Fig. 4).

Nodularia spumigena CCY9414 and *Nodularia harveyana* SAG 44.85 exhibited significantly raised volume specific N₂ fixation rates under HC culture conditions, as observed for *N. spumigena* CCY9414 at 548 ppm CO₂ by Wannicke et al. (2012). Czerny et al. (2009) reported negative effects of HC on cell specific N₂ fixation rates for *Nodularia spumigena* IOW-2000/1 at 16 °C, whereas Eichner et al. (2014) found no significant changes in the same nodularin producing species exposed to elevated CO₂ through continual bubbling of the cultures. Whether these inconsistencies reflect differences in species selection, culture conditions or method of monitoring of N₂ fixation rates can only be determined with repeat experiments under the identical conditions, preferably related to the ecological environment being investigated. A review of published data of N₂ fixation rates in relation to CO₂ gave

evidence for a global positive but non-significant mean effect size for heterocystous species from marine, brackish, and limnic environments (Wannicke et al. 2018b).

When combined with the C assimilation data previously presented, we propose that both the *Nodularia spumigena* strains are capable of immediately responding to elevated CO₂ levels and rapidly increasing their NP rates (Fig. 2). Additionally, they are capable of increasing their biomass with respect to particulate to PON (Supplementary Fig. 7) in the system under HC conditions, thereby making a significant contribution to the primary productivity in the system. *Nodularia harveyana* SAG 44.85 appears to be able to regulate NP under HC conditions (Fig. 2), a trait essential for survival in the organic rich benthic zone (Haynert et al. 2012), but still exhibits a significant contribution to the PON (Supplementary Fig. 7) at the elevated CO₂ conditions investigated in this study.

Significantly, the average N₂ fixation rate of the *Nostoc punctiforme* sp. 73.1 at both LC and HC was 6–17 times lower than that recorded for the *Nodularia* species investigated, although the low number of repetitions prevents a generalisation of the observed trend (Fig. 4).

This study supports the observation of phenotypic plasticity of carbon fixation rates observed for aquatic freshwater *Microcystis* cultures grown under elevated CO₂ conditions of 1000 ppm (Ji et al. 2020). While the *Nostoc* species responded to HC with increased NP rates (Fig. 2), *Nostoc punctiforme* sp. 73.1 most likely did not invest in the highly energy demanding process of N₂ fixation (Fig. 4) under N limitation as observed for the aquatic *Nodularia* species studied. To speculate on a general pattern, however, N₂ fixation measurements have to be repeated for all *Nostoc* species. The PON results (Supplementary Fig. 7) also suggest that *Nostoc* sp. sp. 73.1 would not increase its contribution to N availability in its direct vicinity, thereby possibly offering an explanation as to the overall reduction in Cyanobacterial biomass observed in dryland soilcrusts exposed to HC of 550 ppm for 10 years (Steven et al. 2012). This negative effect of exposure to HC highlights the complexity of dryland biocrust systems and their response to climate change (Reed et al. 2016) may have been the result of reduced BNF and supply of PON to the system. In contrast, an increase in N₂ fixation was observed in earlier studies in cultures of the *Nostoc punctiforme* CPCC41 when grown at elevated CO₂ levels of 940 ppm, about half of the CO₂ used in this investigation (Lindo et al. 2017). Further examination of the survival strategies of these important terrestrial primary producers will offer greater insights into the nutrient partitioning and growth strategies, especially under elevated atmospheric CO₂ levels. Additionally, further research into the phenotypic plasticity of carbon fixation within the complex

filamentous diazotrophs studied here is crucial to understand the effects of climate change on Cyanobacterial primary productivity under future climate change scenarios.

Conclusion

Our study demonstrates species and strain specific variations to elevated atmospheric CO₂ levels. Interestingly, our data suggests that nodularin producers have, on average, higher NP rates than non-nodularin producers under HC conditions, with lower respiration rates. HC growth conditions induce increases in BNF rates and PON levels per volume of cultures of *Nodularia spumigena* CCY9414 and *N. harveyana* SAG 44.85 species, while *Nostoc* BNF rates are seemingly unaffected. Unexpectedly, the combined BNF of all *Nostoc* sp. sp. 73.1 determined for LC and HC are significantly lower than those for all *Nodularia* species tested.

A correlation was observed between HC growth conditions and a decrease in nodularin production under diazotrophic conditions for *Nodularia* CCY9414 and *Nostoc* sp. sp. 73.1 (Fig. 3), with *Nostoc* sp. 73.1 showing increased nodularin content under diazotrophic conditions and *Nodularia spumigena* CCY9414 under LC conditions. Future studies using similar toxin and non-toxin producing Cyanobacteria for which genomic sequence data exists, need to be undertaken under identical conditions to further elucidate the effects of elevated CO₂ on Cyanobacterial cellular metabolism, and the role of secondary metabolites, like nodularin, in mediating the cellular responses to future climate change conditions. This study would suggest that toxin-producing diazotrophs may be less advantaged under current climate change predictions in diazotrophic conditions, due to impaired N₂ fixation under elevated CO₂ conditions, when compared with similar non-toxin producing species of Cyanobacteria. On the other hand, a higher positive response in NP may outbalance this effect at elevated CO₂.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00027-021-00788-6>.

Acknowledgements N.W. thankfully acknowledges the financial support by the Project BIOACID of the German Federal Ministry of Education and Research [BMBF, FKZ 03F0728F]. M. G. funded by the German Research Foundation [DFG GE 2558/3-1 under the SPP1833]. We wish to convey our gratitude to B. Büdel, C. Colesie and E. Neuhäus (TU Kaiserslautern, Germany) for providing experimental facilities and expertise, and to Iris Liskow (The Leibniz Institute for Baltic Sea Research, Germany) for determination of stable isotopes and POM concentrations.

Funding Open Access funding enabled and organized by Projekt DEAL.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

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