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RESEARCH ARTICLE

Atmospheric CO_2 availability induces varying responses in net photosynthesis, toxin production and N_2 fixation rates in heterocystous filamentous Cyanobacteria (*Nostoc* and *Nodularia*)

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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 N_2 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 CO_2 availability on growth, carbon and N_2 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 CO_2 conditions.
- There was a direct correlation between elevated CO_2 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 CO_2 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 CO_2 levels.

This study is the first to provide information on NP and nodularin production under elevated atmospheric CO_2 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 CO_2 (2000 ppm)
LC	Low CO_2 (~440 ppm)
POC	Particulate organic carbon
PON	Particulate organic nitrogen
NP	Nett 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 CO_2 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 CO_2 and generates 2 molecules of 3-phosphoglycerate (3PGA) which is further processed in the Calvin-Benson-Bassham (CBB) cycle to produce ribulose-1,5-biphosphate and glutamate. In order to reduce undesirable oxygenase activity, Cyanobacteria have evolved the carbon concentrating mechanism (CCM) to increase the effective concentration of CO_2 around the Rubisco active site by up to 1000-fold (Price 2011). CO_2 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 CO_2 converting enzyme, NDH-I_4, as well as the low flux, high affinity NDH-I_3 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 HCO_3^- 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 pCO_2 (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 CO_2 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 CO_2 (Ji et al. 2020). The maximum CO_2 uptake rate of *Microcystis* grown at 1000 ppm CO_2 was increased 1.5–1.8 times compared to the low CO_2 control cultures, suggesting that elevated CO_2 conditions may stimulate Cyanobacterial bloom growth (Ji et al. 2020). Furthermore, by reducing the levels of dissolved CO_2 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 CO_2 levels and eutrophication especially of the freshwater lakes are potential drivers of this phenomenon. Approximately a third of all anthropogenic CO_2 released dissolves in the oceans, reducing the pH by increasing the partial pressure of CO_2, accompanied by a smaller relative increase in HCO_3^- and a decrease in CO_3^{2-} (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 CO_2 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 N_2 fixation rates in *Trichodesmium*, with the reduced N_2 fixation rates corresponding to reduced nitrogenase efficiency at lower pH (Kranz et al. 2011). Exposing cultures of the freshwater diazotroph, *Nostoc muscorum,* to raised HCO_3^- concentrations under diazotrophic conditions resulted in enhanced growth, O_2 and pigment production and nitrogenase activities (Bhargava et al. 2013). The brackish diazotroph, *Nodularia spumigena* sp. KAC12, when grown at elevated CO_2 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 CO_2 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 CO_2 (~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 CO_2 levels on Cyanobacterial BNF, and highlighted the diversity in observed responses of marine Cyanobacterial species to elevated atmospheric CO_2. 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 *Crocosphaera.* Very few studies were published on filamentous heterocystous *Nodularia*, *Calothrix* and *Anabaena* (alias: *Dichlospermum*) species (reviewed by Wannicke et al. 2018b). A more recent study suggested that growth of the diazotrophic *Dolichospermum circinale* might benefit from increased CO_2 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 cryptogrammic covers, including Cyanobacteria that contribute

a significant amount to global net primary productivity (Elbert et al. 2012). Specifically, it is estimated that N_2 fixation by cryptogrammic 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 CO_2 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 CO_2 (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 CO_2 (1500 ppm) in moist conditions when compared to mats grown at 350 ppm CO_2 (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% cryptogrammic 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 CO_2 levels (Reed et al. 2016).

Given the evolutionary history of Cyanobacteria already existing under raised CO_2 levels, researchers (Gehringer 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 CO_2 proposed by current climate change scenarios, particularly in eutrophic waters (Ma et al. 2019). Especially toxin producing Cyanobacteria capable of fixing atmospheric N_2 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 (Gehringer 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 Gehringer and Wannicke 2014; Visser et al. 2016; Buratti et al. 2017). Raised temperatures and elevated CO_2 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-Shehawy 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 CO_2 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 (Beversdorf et al. 2013) and increased toxin production (Horst et al. 2014). Inorganic nitrogen limitation was thought to induce a shift to N_2 fixing, diazotrophic Cyanobacteria, thereby increasing organic N availability and a subsequent increase in toxin production (Posch et al. 2012; Gehringer and Wannicke 2014). Recent investigations of bloom dynamics in Lake Müggelsee suggest that the predominant Cyanobacterial diazotrophs, *Aphanizomenon* sp. and *Anabaena* sp (*Dichlospermum* 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 CO_2 to support their continued growth (Paerl and Huisman 2009) with CO_2 availability often limiting bloom growth, a restriction that could be removed under increased atmospheric CO_2 levels. Only aquatic Cyanobacteria carrying the high flux, low affinity BicA HCO_3^- receptor were able to benefit from elevated CO_2 levels and increase their growth rates (Sandrini et al. 2015; 2016; Visser et al. 2016).

Most studies on elevated CO_2 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 CO_2 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 pCO_2 to 1450 ppm pCO_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*CO2, 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 pCO_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 *Aphanizomninaceae*, 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 entophytum* sp. C1.8 (Gehringer 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).

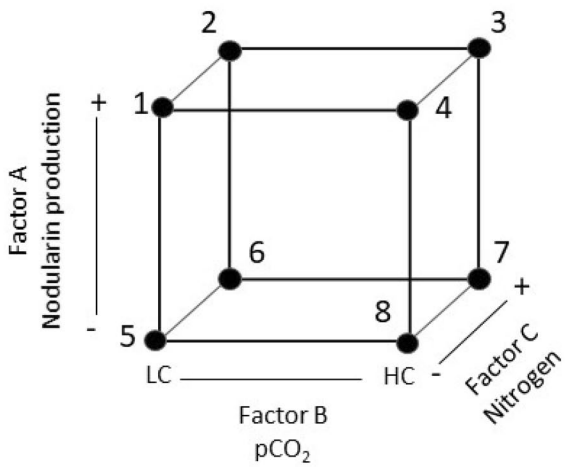
The terrestrially isolated *Nostoc* species were maintained since their isolation on the nitrogen free medium, $BG11_0$, medium with ferric ammonium citrate replaced with ferric citrate (Gehringer et al. 2010), thereby ensuring their ability to fix nitrogen was not lost. Three months prior to this study, 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 T_{175} 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 entophytum* 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 Gehringer 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 µmol photons m^{-2} 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),

(a) Species and experimental characteristics

Species	N_2 fixation rate assessed	Toxin producer	Factorial design see b) Growth and physiology	Factorial design see b) Nodularin analysis
Nostoc punctiforme sp. 73.1	✓	✓	1,2,3,4	1,2,3,4
Nostoc punctiforme sp. 40.5	-	-	6,7	/
Nostoc muscorum sp. 65.1	-	✓	2,3	1,2,3,4
Nostoc entophytum sp. C1.8	-	-	6,7	/
Nodularia spumigena CCY9414	✓	✓	1,4	1,2,3,4
Nodularia spumigena NSBL206	✓	-	5,8	/
Nodularia harveyana 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	-

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 CO_2 treatment (Factor B) with present day levels (440 ppm—"Low CO_2" LC) and elevated CO_2 (2000 ppm- "High CO_2"-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**)

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 N_2 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 OD_{650} 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 OD_{650} 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	*Nostoc sp.**		●		2	2
		Nodularia sp.		●		2+2	4
	HC	*Nostoc sp.**		●		2	2
		Nodularia sp.		●		2+2	4
Incubation and sampling in 96 well- microtiter plates							
Growth based on OD_{650} **		LC	Read on days 3 – 7 and 10, 11, 12 and 14, 6 replicates each				
		HC					
Incubation and sampling in cell culture flasks							
Growth based Chl a.		LC	Sampling at day 3, 5, 7, 10, 12, 14 3 replicates each				
		HC					

*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 entophytum* 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. T_{75} 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 ± 2 µmol kg^{-1}. Total carbon (C_T) and pCO_2 in the growth media were calculated using CO2SYS (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 (Hermann and Gehringer 2019).

Net CO_2 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 CO_2 uptake determinations (between 1 and 4 h after sampling) by means of CO_2 gas exchange measurements (GFS 3000, WALZ, Effeltrich, Germany). Bacterial covered filters of LC acclimated cultures were placed in the sample cuvette and CO_2 uptake determined at 80% humidity at 440 ppm (Herrmann and Gehringer, 2019), while for HC acclimated cultures measurements was done at 1500 ppm CO_2 (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. CO_2 assimilation rates were determined at 500 µmol photons m^{-2} 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 CO_2 minus the CO_2 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 CO_2 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 OD_{665} was determined (Lambda 35 UV/VIS spectrometer, Perkin-Elmer). Chlorophyll *a* content was calculated using the equation: Chl *a* µg $ml^{-1} = OD_{665} \times 12.7$ (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 [ng nodularin. µg dry $weight^{-1}$].

Particulate organic matter and N_2 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). N_2 fixation activity was determined by incubating cultures in two replicates per treatment with bubble addition of $^{15}N–N_2$ enriched gas (99% $^{15}N_2$) 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. $\ln RR = \left(\frac{\bar{x}_T}{\bar{x}_C}\right)$ for net photosynthesis, dark respiration, growth and N_2 fixation in selected Cyanobacteria. Here lnRR 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 lnRR 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 lnRR values were combined to give a mean effect size (i.e. Cohen's *d*). A weight was assigned to each lnRR obtained from individual species which was inversely proportional to its sampling variance (DerSimonian and Laird 1986) as represented by the following equation: $d = \frac{\bar{x}_T - \bar{x}_C}{\text{SampleSDpooled}}$. 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 pCO_2 in the growth media of cultures incubated at HC in the *Nostoc* cultures was determined to be 1987 ± 42 µatm for N-free media, while the pCO_2 in the LC treatment was 293 ± 38 µatm. *Nodularia* cultures displayed a mean pCO_2 of 1701 ± 83 µatm in N-replete brackish seawater medium (Suppl. Table 1). Control cultures at LC conditions revealed significantly reduced pCO_2 availability at 232 ± 26 µatm. Control medium at HC was 2728 ± 323 µatm and 2203 ± 405 µatm 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 entophytum* 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 entophytum* 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 diazotophic (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 diazotophic (534.0 ± 343.0 µg ml^{-1}) and N-replete (622.0 ± 333.7 µg ml^{-1}) conditions.

Effect of elevated CO_2 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 ($p \leq 0.005$) 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 ($F = 36.3$, $n = 92/22$, $p \leq 0.001$). 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).

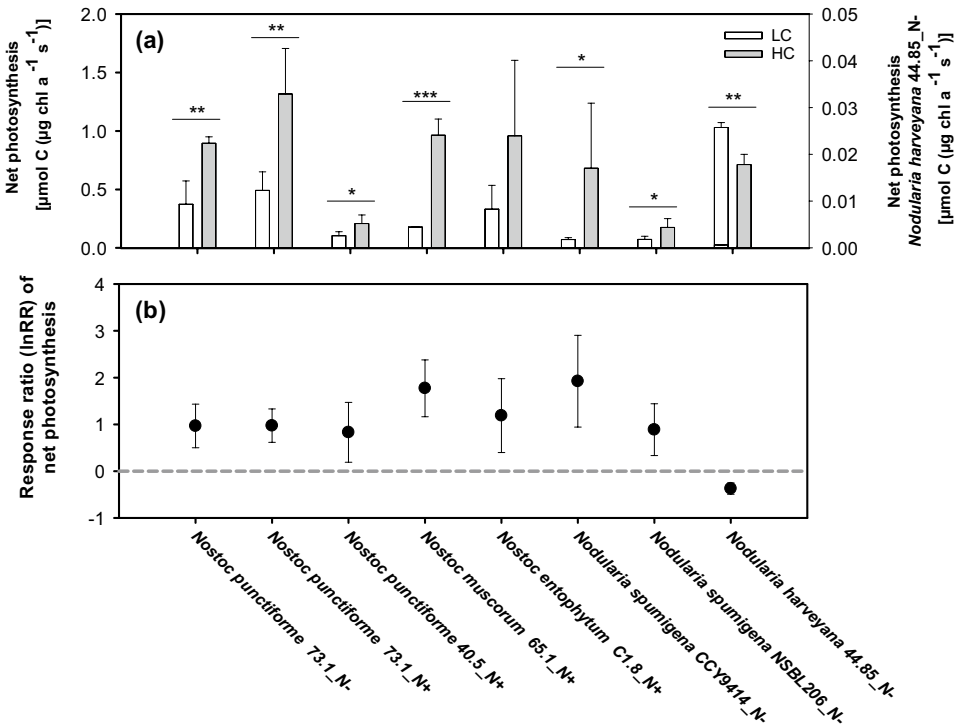


Fig. 2 **a** Net photosynthesis (NP) in control cultures (LC-440 ppm) and cultures grown at elevated CO_2 (HC-2000 ppm). Bars represent mean and standard deviation of four measurements (day 7 + day 14). Second y-axis (RHS) refers to *Nodularia harveyana* SAG 44.85. Significant differences between measurements are indicated by $*p \leq 0.05$, $** p \leq 0.005$, $***p \leq 0.001$ according to repeated -measure ANOVA and **b** Response ratio of net photosynthesis was calculated by determining 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 CO_2 treatment (i.e. $RR = 0$). If the CI crossed the 0 response line, the effect of elevated CO_2 is considered as non-significant. Mean and $CI > 0$ indicate a stimulation by elevated CO_2. Mean and $CI < 0$ indicate a negative effect of elevated CO_2

Effect of elevated atmospheric CO_2 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 CO_2 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 CO_2 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 CO_2 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).

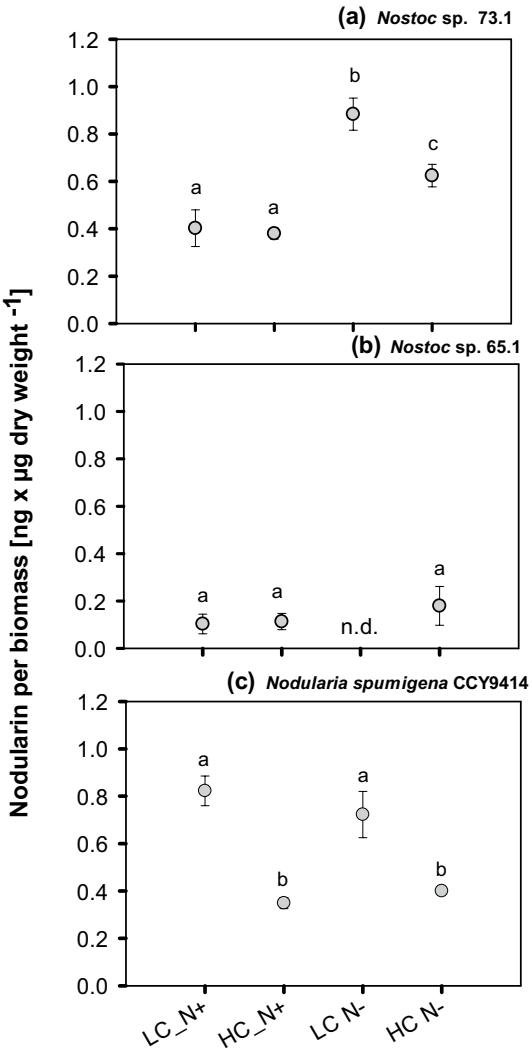


Fig. 3 Total soluble cellular nodularin content per µg dry weight for control cultures (LC-440 ppm) and cultures grown at elevated CO_2 (HC-2000 ppm). Values represent mean and standard deviation determined after 14 days of incubation (n = 3). Significant differences are indicated by different letters to the level of $p \leq 0.05$ according to one-way ANOVA

Effect on N_2 fixation

N_2 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 N_2 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 N_2 fixation in *Nodularia spumigena* NSBL206.

N_2 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 pCO_2 (HC) (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 CO_2 response in nodularin and non-nodularin producer

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

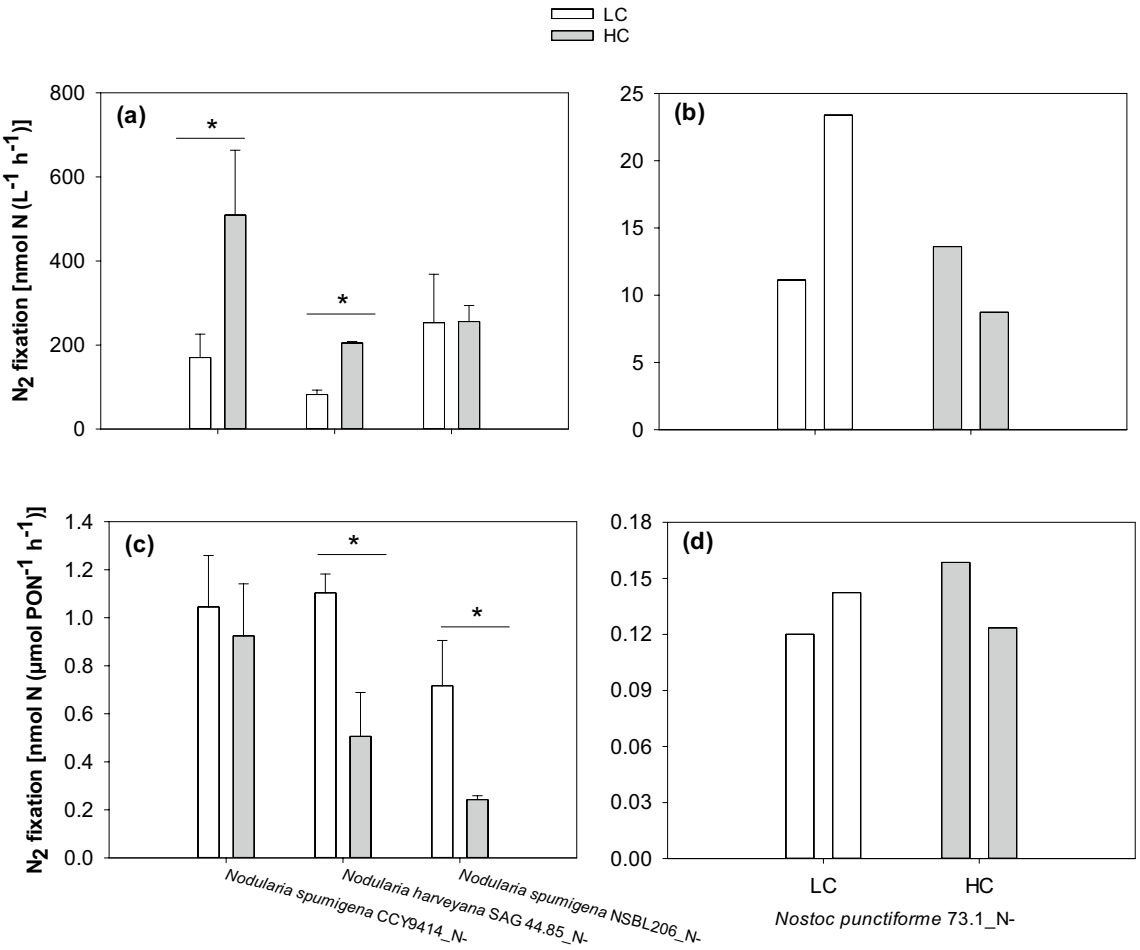


Fig. 4 Volume specific and biomass specific N_2 fixation rates determined for *Nodularia* sp. (**a**, **c**) and *Nostoc punctiforme* sp. 73.1 (**b**, **d**) grown at LC (440 ppm) and HC (2000 ppm). Bars in **a** and **c** represent mean and standard deviation of four replicates, bars in **b** and **d** 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 CO_2 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 CO_2 treatment on growth, while the nodularin producers displayed a significant positive response to HC. Contrasting patterns were visible for N_2 fixation, depending on the mode of normalisation of rates, namely volume or PON. Weighted mean effect sizes of biomass specific N_2 fixation showed opposing directions and a significant difference in the subgroups ($p \leq 0.001$, Student's *t* test) with a significant decrease in N_2 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 N_2 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 CO_2 emission rates are not being reduced as rapidly as desired, suggesting levels of CO_2 which will most likely exceed 1000 ppm by the year 2100 for the worst- case-scenario (RCP8.5) (IPCC 2019).

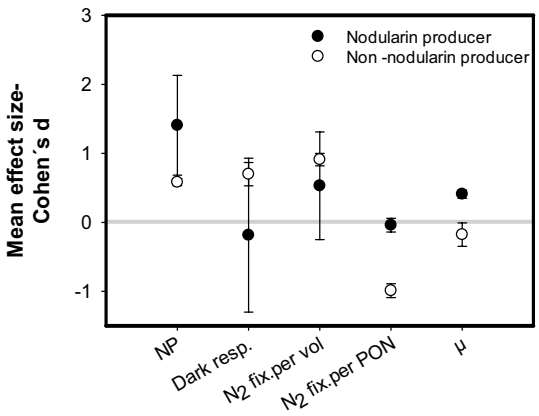


Fig. 5 Weighted mean effect sizes (Cohen's *d*) of net photosynthesis (NP), respiration, growth and N_2 fixation for the two subgroups "Nodularin producer" (black circles, for details on Cyanobacteria see Fig. 1a) and "Non-nodularin producer" (white circles, for details on Cyanobacteria see Fig. 1a). Data is presented as means and 95% confidence interval (CI). The horizontal grey line indicates lack of response to the CO_2 treatment (i.e. mean effect size = 0). If the CI crossed the 0 response line, the effect of elevated CO_2 is considered as non-significant. Mean and CI > 0 indicate a stimulation by elevated CO_2. Mean and CI < 0 indicate a negative effect of elevated CO_2

Recent reviews have summarised the literature regarding the response of marine and freshwater bloom forming Cyanobacteria to elevated CO_2 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 (Gehringer and Wannicke 2014). While numerous studies have been conducted on toxin and non-toxin producing *Microcystis aeruginosa* strains under elevated CO_2 conditions, little data has been accumulated as to the effects on diazotrophic Cyanobacteria, especially terrestrial *Nostoc* species (Gehringer and Wannicke 2014). In this study, the effects of elevated atmospheric CO_2 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 (Cohens'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 Cohens's d plot (Fig. 5), while toxin producers had slightly lower rates. This trend appeared inversed 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 CO_2 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 CO_2 on NP and growth

All aquatic and terrestrial diazotrophic Cyanobacteria investigated in this study fixed atmospheric CO_2 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 CO_2, 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 CO_2. 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% identitity to the *Bic*A gene of *Microcystis aeruginosa* PCC7806, thereby suggesting that it can benefit from increased HCO_3^- in the media and thus, increase its NP rates accordingly (Visser et al. 2016). Gas exchange measurements were used to assess NP (Herrmann and Gehringer 2019) as most Cyanobacteria are known to encode the CO_2 converting enzymes, NDH-I_4 and NDH-I_3 (Visser et al. 2016), necessary for the direct, non-energy demanding conversion of CO_2 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.

Alkalisation of the culture medium of *Nostoc* species grown under N-replete conditions at both HC and LC culture conditions was recorded, whereas alkalisation 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 CO_2 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 CO_2 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 CO_2 in water and the slow equilibrium between CO_2 and HCO_3 (e.g. Ibelings and Maberly 1998). Photosynthesis in biocrusts is also often limited by CO_2 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 CO_2 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 CO_2 exposure

Intracellular nodularin content showed significant variation dependent on the Cyanobacterium and/ or atmospheric CO_2 content (Fig. 3). *Nostoc* sp. 65.1 appears to constitutively produce nodularin at low levels, independent of medium N content and atmospheric CO_2 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* IMS101cultures exposed to elevated levels of CO_2 (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 N_2 fixation rates under HC culture conditions, as observed for *N. spumigena* CCY9414 at 548 ppm CO_2 by Wannicke et al. (2012). Czerny et al. (2009) reported negative effects of HC on cell specific N_2 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 CO_2 through continual bubbling of the cultures. Whether these inconsistencies reflect differences in species selection, culture conditions or method of monitoring of N_2 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 N_2 fixation rates in relation to CO_2 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 CO_2 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 CO_2 conditions investigated in this study.

Significantly, the average N_2 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 CO_2 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 N_2 fixation (Fig. 4) under N limitation as observed for the aquatic *Nodularia* species studied. To speculate on a general pattern, however, N_2 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 N_2 fixation was observed in earlier studies in cultures of the *Nostoc punctiforme* CPCC41 when grown at elevated CO_2 levels of 940 ppm, about half of the CO_2 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 CO_2 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 CO_2 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 CO_2 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 N_2 fixation under elevated CO_2 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 CO_2.

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Compliance with ethical standards

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

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