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Elevated CO_2 significantly increases N_2 fixation, growth rates, and alters microcystin, anatoxin, and saxitoxin cell quotas in strains of the bloom-forming cyanobacteria, *Dolichospermum*

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ABSTRACT

The effect of rising CO_2 levels on cyanobacterial harmful algal blooms (CHABs) is an emerging concern, particularly within eutrophic ecosystems. While elevated pCO_2 has been associated with enhanced growth rates of some cyanobacteria, few studies have explored the effect of CO_2 and nitrogen availability on diazotrophic (N_2-fixing) cyanobacteria that produce cyanotoxins. Here, the effects of elevated CO_2 and fixed nitrogen (NO_3^-) availability on the growth rates, toxin production, and N_2 fixation of microcystin, saxitoxin, and anatoxin-a - producing strains of the genus *Dolichospermum* were quantified. Growth rates of all *Dolichospermum* spp. were significantly increased by CO_2 or both CO_2 and NO_3^- with rates being highest in treatments with the highest levels of CO_2 and NO_3^- for all strains. While NO_3^- suppressed N_2 fixation, diazotrophy significantly increased when NO_3^--enriched *Dolichospermum* spp. were supplied with higher CO_2 compared to cultures grown under lower CO_2 levels. This suggests that diazotrophy will play an increasingly important role in N cycling in CO_2-enriched, eutrophic lentic systems. NO_3^- significantly increased quotas of the N-rich cyanotoxins, microcystin and saxitoxin, at ambient and enriched CO_2 levels, respectively. In contrast, elevated CO_2 significantly decreased cell quotas of microcystin and saxitoxin, but significantly increased cell quotas of the N-poor cyanotoxin, anatoxin. N_2 fixation was significantly negatively and positively correlated with quotas of N-rich and N-poor cyanotoxins, respectively. Findings suggest cellular quotas of N-rich toxins (microcystin and saxitoxin) may be significantly reduced, or cellular quotas of N-poor toxins (anatoxin) may be significantly enhanced, under elevated CO_2 conditions during diazotrophic cyanobacterial blooms. Finally, in the future, ecosystems that experience combinations of excessive N loading and CO_2 enrichment may become more prone to toxic blooms of *Dolichospermum*.

1. Introduction

Harmful cyanobacterial blooms (CHABs) are globally recurring in greater frequency and intensity, particularly in lentic systems that exhibit excessive nutrient loading, or eutrophication (O'Neil et al., 2012; Gobler et al., 2016; Paerl et al., 2016). While phosphorus is traditionally assumed to solely limit freshwater primary production (Smith, 1983; Schindler et al., 2008; Smith, 2016), recent evidence indicates that CHAB taxa can also exhibit fixed nitrogen (N) limitation (Davis et al., 2015; Harke et al., 2016; Qian et al., 2017). Climatic warming can also enhance the growth of CHAB taxa under eutrophic conditions, as cyanobacteria's maximum growth rates occur at higher temperatures than those of eukaryotic algae (Robarts and Zohary, 1987; Paerl and Paul, 2012; Jankowiak et al., 2019). While the benefit of rising temperatures for freshwater CHABs is strongly supported by previous studies (Joehnk et al., 2008; Paerl and Paul, 2012; Mullin et al., 2020), the effect of elevated atmospheric carbon dioxide (CO_2) levels on taxa that cause such blooms is considerably less well known, particularly for diazotrophic cyanobacteria.

Elevated atmospheric CO_2 levels were, until recently, thought to have only a minor impact on freshwater systems because of their tendency to exhibit CO_2 supersaturation relative to the atmosphere (Cole et al., 1994; Hasler et al., 2016). Enhanced primary productivity in freshwater systems due eutrophication and rising temperatures can cause dramatic fluctuations in inorganic carbon inventories, however, subsequently resulting in CO_2 undersaturation relative to the

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atmosphere, particularly during warmer periods coincident with CHABs (Balmer and Downing, 2011). Considering this, CO_2 availability could play a significant role in promoting freshwater primary productivity, at least under eutrophic (fixed N and P - replete) conditions (Jannson et al., 2012; Visser et al., 2016; Symes and van Ogtrop, 2019), including that of the cyanobacterial genus *Microcystis* (Verspagen et al., 2014; Ma et al., 2019; Ji et al., 2020). *Microcystis* strains may also decrease production of the hepatotoxin microcystin under elevated CO_2 levels (Van de Waal et al., 2011; Yu et al., 2015; Liu et al., 2016), while N-replete conditions may reverse this effect (Van de Waal et al., 2009). These changes demonstrate that microcystin production by *Microcystis* can be sensitive to the stoichiometry of exogenously available C and N (Downing et al., 2005; Van de Waal et al., 2010; Harke and Gobler, 2013). However, the combined effect of elevated CO_2 and fixed N enrichment on the growth rates and toxin production of other freshwater cyanobacteria, particularly diazotrophs, is considerably less well understood.

Most freshwater diazotrophic cyanobacteria belong to Nostocales: an order of freshwater and brackish cyanobacteria that, under fixed N-limiting conditions, develop heterocysts that anaerobically convert dinitrogen (N_2) gas into ammonia via the enzyme nitrogenase (Wolk et al., 1994; Hoffman et al., 2014). CHABs dominated by diazotrophic taxa are common in eutrophic systems and are increasing in intensity (Sukenik et al., 2012; Li et al., 2016; Budzyńska et al., 2019). There is also evidence that these taxa may benefit from rising CO_2 levels via the relief of inorganic carbon limitation (Endres et al., 2013; Pierangelini et al., 2014; Willis et al., 2019). Little is known, however, about the effects of changing CO_2 and fixed N levels on Nostocales growth and cyanotoxin production. The only studies to characterize the effects of CO_2 and fixed N on Nostocales taxa are those of the brackish cyanobacterium *Nodularia spumigena*. Lab-based studies reported an enhanced N_2 fixation rate under a high CO_2 regime and a negative correlation between dissolved inorganic nitrogen (DIN) and pCO_2 (Wannicke et al., 2012). Furthermore, similar experiments with other *Nodularia* strains indicated that elevated CO_2 levels (2000 μatm) enhanced photosynthetic activity and N_2 fixation as well as reduced cellular concentrations of the N-rich (Van de Waal et al., 2014) hepatotoxin, nodularin (Wannicke et al., 2021). Still, there are many unknowns regarding how freshwater cyanobacterial growth, diazotrophy, and toxin production are affected by an elevated CO_2 regime and fixed N availability.

Dolichospermum (formerly *Anabaena*) is a genus of Nostocales that is capable of producing a variety of highly potent toxic metabolites including microcystin as well as the neurotoxins anatoxin and saxitoxin (O'Neil et al., 2012; Li et al., 2016; Capelli et al., 2017; Österholm et al., 2020). Studies have reported enhanced growth and microcystin cellular quotas in *Dolichospermum* sp. BIR257 (Brutemark et al., 2015) as well as the dominance of members of this genus within natural cyanobacterial communities (Shi et al., 2017) under elevated CO_2 conditions. Only one study, however, has characterized the effect of CO_2 and fixed N on *Dolichospermum*, which found a significant increase in heterocyst density as CO_2 levels increased in the freshwater medium BG11 (Kang et al., 2005), which includes 17.6 mM NO_3^- (Stainer et al., 1971). This observation, coupled with the significant reduction in NO_3^-uptake and reductase activity in response to CO_2 enrichment (Kang et al., 2005), suggests that N_2 fixation in *Dolichospermum* taxa might increase in response to elevated CO_2 levels even under fixed N-replete conditions, and that nitrogenase activity is sensitive to the C:N ratio. Ultimately, however, N_2 fixation and toxin production have never been measured under multiple CO_2 regimes in *Dolichospermum* taxa.

The purpose of this study, therefore, was to quantify the effects of CO_2 enrichment and fixed N availability on the growth, toxin quota, and N_2 fixation on microcystin, anatoxin, and saxitoxin - producing strains of *Dolichospermum*. It was hypothesized that these *Dolichospermum* strains would exhibit significantly enhanced growth and N_2 fixation under an elevated CO_2 regime relative to those grown under ambient CO_2 levels, regardless of whether they are amended with fixed N. It was also hypothesized that the cellular quotas of N-rich toxins such as microcystin and saxitoxin will be significantly reduced under an elevated CO_2 regime relative to ambient CO_2 conditions. Conversely, as anatoxin is N-poor (Van de Waal et al., 2014), it was also hypothesized that elevated CO_2 conditions would significantly increase anatoxin cellular quotas. This is the first study to characterize growth, N_2 fixation, and the production of multiple cyanotoxins under multiple CO_2 and fixed N regimes in *Dolichospermum*.

2. Materials and methods

2.1. Experimental setup

Dolichospermum sp. 90, a microcystin (MC) producer, *Dolichospermum* sp. 54, an anatoxin (ATX-A) producer, and *D. circinale* ACBU02, a saxitoxin (STX) producer (Rouhiainen et al., 1995; Pereyra et al., 2017), were used for experimentation. Strains sp. 54 and sp. 90 were isolated from lakes in Finland, while strain ACBU02 was isolated from Australia (Rouhiainen et al., 1995; Pereyra et al., 2017). All cultures were maintained in freshwater medium BG11 (Stainer et al., 1971) without NO_3^- (BG11-N) in autoclaved 8 L containers at 21 °C as well as a 14:10 light/dark cycle and positioned so that light levels were 60 ± 10 μmol photons m^{-2} s^{-1} which supported maximal growth rates Vessels were bubbled with HEPA - filtered air to reduce clumping and light limitation, and to increase dissolved inorganic carbon (DIC) availability in the media (Gattuso and Lavigne, 2009; Wynn-Edwards et al., 2014). Sterile BG11-N was added to 1 L Erlenmeyer flasks, after which sodium nitrate ($NaNO_3$) was added to half of them (1 mM NO_3^-). A total of 24 flasks were used for the *Dolichospermum* sp. 90 experiment and included 4 treatments: cultures grown under ambient CO_2 conditions and amended with (-CO_2+N) or deprived of (-CO_2–N) NO_3^-, and cultures grown under extremely enriched CO_2 conditions and amended with (+++CO_2+N) or deprived of (+++CO_2–N) NO_3^- ($n = 6$). Experiments with *Dolichospermum* sp. 54 and *D. circinale* ACBU02 included, along with ambient (-CO_2) and extremely (+++CO_2) enriched CO_2 treatments with or deprived of NO_3^-, intermediate elevated CO_2 treatments. These cultures were grown under moderately enriched CO_2 conditions and amended with (+CO_2+N) or deprived of (+CO_2–N) NO_3^-, and cultures grown under highly enriched CO_2 conditions and amended with (++CO_2+N) or deprived of (++CO_2–N) NO_3^-. Experiments with the ATX-A and STX - producing strains included a total of 40 flasks and 8 treatments ($n = 5$). The control treatment in all experiments were those *Dolichospermum* spp. grown in BG11-N under ambient CO_2 (-CO_2–N) conditions.

These flasks were then placed in an incubator set at 21 °C as well as a 14:10 light/dark cycle and positioned so that light levels were 60 ± 10 μmol photons m^{-2} s^{-1}. Flasks were bubbled for 1–2 days at their corresponding CO_2 levels, which was achieved via bubbling with ambient air or a mixture of ambient air and compressed 5% CO_2 in air using Masterflex flowmeters (Cole-Parmer) and PVC manifolds to maintain a constant flow rate of ~200 mL min^{-1}. The method of CO_2 delivery, or whether CO_2 - enriched air was directly bubbled into or aerated above water, does not significantly affect the degree of change in pH and DIC concentration (Brown et al., 2020). Immediately prior to inoculation, pH was measured in flasks using an Orion Star A121 portable pH meter (ThermoFisher Scientific). The pH of pre-inoculated flasks roughly fall within the range of pH (Supplemental Table 1) measured in 131 eutrophic US lakes as well as eutrophic lentic systems on other continents (6.26 - 10.86) (Balmer and Downing, 2011; Zagarese et al., 2021).

The media of two "mock" flasks from each treatment (sp. 90 $n = 8$; sp. 54 and *D. circinale* ACBU02 $n = 16$) was transferred without bubbling to 300 mL acid-washed (10% HCl) borosilicate glass BOD bottles (VWR) via a Masterflex L/S Peristaltic Pump (Cole-Parmer) using deionized (DI) water - rinsed Tygon rubber tubing until the contents of the bottle had overflowed (Bockmon and Dickson, 2014; Bockmon and Dickson, 2015). Samples were preserved in 1% mercuric chloride, sealed with Apiezon vacuum L-type grease, and stored at 4 °C prior to DIC quantification

described below. Cell densities and in vivo chlorophyll-*a* (chl-*a*) fluorescence of pre-experimental *Dolichospermum* cultures were determined immediately prior to inoculation using a gridded 1mm^2 Sedgewick Rafter and a Walz PHYTO-PAM-II (Klughammer and Schreiber, 2015), respectively. A fraction of the sterile media from the remaining flasks of each treatment (sp. 90 $n = 4$; sp. 54 and *D. circinale* ACBU02 $n = 3$) was removed and replaced with pre-experimental *Dolichospermum* culture to a cell density of ~10^5 cells mL^{-1} (sp. 90 = 5.0×10^5 cells mL^{-1}; sp. 54 = 2.5×10^5 cells mL^{-1}; *D. circinale* ACBU02 = 1.0×10^5 cells mL^{-1}). +N flasks were then re-amended with NO_3^- to bring the final concentration back to 1 mM.

2.2. Experimental design and monitoring

To allow *Dolichsopermum* spp. to physiologically acclimate to experimental conditions, cultures were grown semi-continuously for several weeks (sp. 90 = 8 weeks; sp. 54 and *D. circinale* ACBU02 = five weeks), with cultures reaching late logarithmic phase (usually ~7 days) diluted back down to their initial biomass in new flasks containing fresh media. Fixed N-amended cultures were re-amended with 1 mM $NaNO_3$. During experiments, chl-*a* fluorescence was used as a proxy for cell density, as there was a significant (F-test; $p < 0.001$) positive correlation between the two parameters for all three taxa (Supplemental Figure 1). For the first several weeks, pH and chl-*a* values were measured every other day. In the final weeks of experimentation, additional physiological parameters were measured, and the means by which they were measured are described in the following section.

2.3. Cell quantification and specific growth rate

During the final one- to two-week period of each experiment, in addition to chl-*a* and pH being measured daily, cells were preserved in Lugol's iodine every other day and enumerated under an inverted Nikon Eclipse TS100 microscope using a gridded 1 mm^2 Sedgewick Rafter counting chamber, with care taken to manually focus on each cell of each chain in cases where they took on a three-dimension structure. At least 200 cells were counted per sample and replicated counts of the same sample with this approach provided a precision of ± 7%. Maximum growth rates [$\mu_{max} = (\mathrm{Ln}(N_2/N_1))/(t_2 - t_1)$], measured in units of day^{-1}, were determined from cell densities, where the change in cell density (N) between days t_2 and t_1 was calculated (Guillard, 1973). Toxin concentrations (μg L^{-1}) and N_2 fixation rates (μmol N_2-fixed L^{-1} day^{-1}) were normalized to biomass (cells mL^{-1}) to determine cellular toxin quotas and the amount of N_2 fixed per cell, respectively. Heterocysts were also distinguished from vegetative cells (Thiel and Pratte, 2001; Masukawa et al., 2017) and quantified to determine the heterocyst-to-total cell (H:TC) ratio, with values for each replicate averaged across the sampling period prior to statistical analysis.

2.4. N_2 fixation rate measurements (acetylene reduction assay)

N_2 fixation rates were also measured every other day using the acetylene reduction method (Capone, 1993; Hardy et al., 1973), as one molecule of N_2 is fixed for every four ethylene (C_2H_4) molecules produced in *Dolichospermum* (Jensen and Cox, 1983). Acetylene was made by reacting 7 g of lab-grade calcium carbide (Fisher Scientific) with 700 mL deionized water (Hyman and Arp, 1987), with the resulting gas collected in Supelco Tedlar bags. Immediately prior to sample analysis, C_2H_4 standards used to quantify the amount of N_2 fixed in samples over time were made by injecting 1% ethylene in N_2 (Airgas) into empty 21 mL vials sealed with magnetic screw-caps. Acetylene (C_2H_2) was injected into the headspace of vials containing 10 mL of sample, then placed back into the incubator with experimental cultures for 3–4 h, after which a portion of the headspace was withdrawn with a gas-tight syringe and injected into a Trace 1310 Gas Chromatograph coupled with a TriPlus 500 GC Headspace Autosampler (Thermo Scientific). The amount of ethylene produced during the acetylene incubation period was visualized and quantified using Chromeleon Chromatography Data System (CDS) software (Version 7.3). The amount of N_2 fixed at a particular time point was normalized to cell density with values for each replicate averaged across the sampling period prior to statistical analysis.

2.5. Cyanotoxin analysis and quantification (MC, ATX-A, and STX)

For the *Dolichospermum* sp. 90 experiment, whole water samples (1 mL) were collected every other day and stored at −20 °C prior to microcystin analysis using microcystins/nodularins Eurofins Abraxis enzyme-linked immunosorbent assay (ELISA) kits, which utilize antibodies that bind specifically to the 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (ADDA) group of the MC congener microcystin-LR (MC-LR), which includes leucine (L) and arginine (R) as amino acids (James et al., 2010). Immediately prior to analysis, samples were freeze-thawed to physically disrupt cells, subsequently releasing microcystin into the dissolved fraction, then filtered using pre-combusted (450 °C) glass fiber filters (GF/Fs). Values were normalized to cell densities then averaged across all timepoints per replicate prior to statistical analysis. For *Dolichospermum* sp. 54 (ATX-A-producer) and *D. circinale* ACBU02 (STX-producer), 15 mL of culture at the final timepoint were filtered on a combusted GF/F and stored at −20 °C prior to preparation for analysis.

ATX-A was extracted using a modified protocol described previously (Dell'Aversano et al., 2005; EPA, 2015), in which filters were immersed in an acetonitrile:H_2O:formic acid (80:19.9:0.1) mixture and sonicated for 30 min. Samples were re-filtered post-sonication through pre-combusted GF/Fs into glass scintillation vials. A 5 μL of sample was then diluted 200-fold in the same solvent and analyzed via high performance liquid chromatography - tandem mass spectrometry (HPLC-MS/MS) using an Agilent 1260 Infinity II LC system and an Agilent 6495 triple quadrupole mass spectrometer. ATX-A was detected with qualifier and quantifier ions (James et al., 2005) using Agilent MassHunter Qualitative Analysis software (version B.08.00). A known concentration (100 ng mL^{-1}) of ATX-A (National Research Council of Canada) was added to a GF/F and processed the same way as the samples, which indicated ~85% of the compound was recovered. L-phenylalanine-d5 (Cambridge Isotope Laboratories) at a known concentration (1 μg L^{-1}) was also added to each sample to correct for changes in the sensitivity of the HPLC-MS/MS during sample analysis.

STX samples were analyzed at the Bigelow Laboratories for Ocean Sciences in Boothbay, ME, USA, using a previously described protocol (Poulton et al., 2005). Briefly, GF/F filters were completely immersed in cold 0.05 M acetic acid (HOAc), sonicated for 30 min, centrifuged at 3220 RCF x 10 min, and the supernatant filtered through 13 mm, 0.2 μm nylon filters into autosampler vials. Samples were then analyzed using high performance liquid chromatography with post-column oxidation and fluorescence detection (HPLC-PCOX-FLD) according to the instrument settings of AOAC Official Method 2011.02 (Van de Riet et al., 2011). STX congeners, namely carbamates including saxitoxin (STX), gonyautoxin (GTX1–4), and neosaxitoxin (NEO), as well as decarbamoyl analogs (dcGTX3, dcGTX2, and dcSTX), and N-sulfocarbamoyl paralytic shellfish toxin congeners (GTX5, C1, and C2) were quantified. The sum value of all 12 congeners was normalized to cell density to determine total saxitoxin (Total-STX) cellular quotas (Van de Riet et al., 2009; Hattenrath-Lehmann et al., 2017). Cell quotas of Total-STX were also plotted as functions of N_2 fixation rates via linear regression analyses.

2.6. DIC analysis and subsequent carbonate chemistry determination

Samples analyzed for DIC included those from mock flasks preserved immediately prior to the start of experiments, as well as those collected at the final timepoints. For experiments with *Dolichospermum* sp. 90 and *D. circinale* ACBU02, samples were also collected during the water change prior to the final period of sampling. BOD bottles containing

Dolichospermum were filtered through a GF/F glass fiber filter secured in a filter holder using tubing and a peristaltic pump to pass sample without bubbling into a 10% HCl-washed BOD bottle (Bockmon and Dickson, 2014). Analysis of selected samples before and after filtration indicated no significant difference in DIC or pCO_2 levels (data not shown). DIC analysis was performed using a VINDTA 3D delivery system coupled with a UIC Inc. coulometer (model CM5017O) (Johnson et al., 1993; Young and Gobler, 2018). Using pH (NBS scale), temperature, DIC, and orthophosphate values, alkalinity and pCO_2 were calculated using the software program CO2SYS (https://cdiac.ess-dive.lbl.gov/ftp/co2sys/) using the first and second dissociation constants of carbonic acid in freshwater (Millero, 1979). Salinity was assumed to be zero. To confirm the accuracy of inorganic carbon values, DIC and pH values from certified reference material (CRM; Scripps Institution of Oceanography) were measured with every set of samples. Sample analysis proceeded only after >99.8% of the CRM was recovered. Carbonate chemistry values from each replicate in each treatment across all time points were averaged together prior to statistical analysis.

2.7. Statistical analyses

Statistical analyses were performed in R studio (Version 4.0.3), while graphing was done in Microsoft Excel (Version 16.51). For *Dolichospermum* spp. experiments, a two-way analysis of variance (ANOVA) was used to determine whether NO_3^- availability and CO_2 enrichment significantly ($p < 0.05$) affected growth rate, toxin cellular quotas, N_2 fixation, H:TC, and carbonate chemistry. Shapiro-Wilk tests were used to confirm that the data passed normality ($p>0.05$), while Fligner-Killeen (growth rate, N_2 fixation, H:TC, and DIC) and Bartlett's tests (toxin cellular quotas) were used to confirm that the data passed homogeneity of variance ($p>0.05$). Tukey's HSD tests were performed *post-hoc* to determine whether differences among treatments were significant ($p < 0.05$). Linear regression analyses were also performed, with cellular toxin quotas plotted as functions of N_2 fixation to determine whether correlations were significant (F-test; $p < 0.05$).

3. Results

3.1. Effect of CO_2 and NO_3^- on Dolichospermum sp. 90 (microcystin-producer)

Over the course of the experiment with *Dolichospermum* sp. 90, the mean (±SD) pH and pCO_2 values decreased and increased, respectively, at higher CO_2 delivery rates (Table 1). CO_2 enrichment (Two-way ANOVA; $p < 0.01$) and NO_3^- availability (Two-way ANOVA; $p < 0.01$) each significantly increased maximum growth rates (μ_{max} = day^{-1}; Fig. 1A). The growth rates of +++CO_2 (+*N* = 0.46 ± 0.05 day^{-1}; -*N* = 0.36 ± 0.03 day^{-1}) and -CO_2+N (0.35 ± 0.04 day^{-1}) were significantly (Tukey's HSD; $p < 0.05$) faster than those grown under the control conditions (-CO_2-N = 0.18 ± 0.07 day^{-1}). NO_3^- availability significantly, antagonistically interacted with CO_2 enrichment (Two-way ANOVA; $p < 0.001$) to alter N_2 fixation rates (Fig. 2B). -N cultures exhibited N_2 fixation rates (-CO_2-N = 0.02 ± 0.003 pmol N_2-fixed cell^{-1} day^{-1}; +++CO_2-N = 0.02 ± 0.002 pmol N_2-fixed cell^{-1} day^{-1}) that were significantly greater than those of -CO_2+*N* (Tukey's HSD; $p < 0.05$). In addition, CO_2 enrichment led to the N_2 fixation rates of +++CO_2+*N* (0.01 ± 0.002 pmol N_2-fixed cell^{-1} day^{-1}) being an order of magnitude higher than those of -CO_2+N (-CO_2+*N* = 0.001 ± 0.0003 pmol N_2-fixed cell^{-1} day^{-1}; Tukey's HSD; $p < 0.05$). Heterocyst-to-total cell ratios (H:TC) were also antagonistically altered (Two-way ANOVA; $p < 0.001$) by NO_3^- availability and CO_2 enrichment (Fig. 1C). +++CO_2+*N* cultures exhibited a significantly (0.03 ± 0.001; Tukey's HSD; $p < 0.05$) higher H:TC than -CO_2+*N* cultures (0.02 ± 0.003). Consequently, N_2 fixation rates were significantly correlated with H:TC (F-test; $R^2 = 0.80$, $p < 0.001$). Microcystin-LR (MC-LR) equivalent (MC) cell quotas significantly increased due to NO_3^- enrichment (Two-way ANOVA; $p < 0.01$) and decreased due to CO_2 enrichment (Two-way ANOVA; $p < 0.001$) with no interaction between the two independent variables (Fig. 1D). MC quotas in -CO_2+*N* cultures were significantly (Tukey's HSD; $p < 0.05$) higher (0.42 ± 0.04 pg MC-LR equiv. cell^{-1}) than those of -CO_2–N (0.32 ± 0.04 pg MC-LR equiv. cell^{-1}), though CO_2 enrichment significantly reduced quotas relative to -CO_2 cultures (Tukey's HSD; $p < 0.05$; Fig. 1D). MC quotas were also significantly negatively correlated with growth rates (F-test; $R^2 = -0.40$, $p < 0.01$) and N_2 fixation rates (F-test; $p < 0.01$, $R^2 = -0.60$, Fig. 1E) across treatments.

Table 1
Carbonate chemistry of *Dolichospermum* sp. 90 cultures grown under ambient (-) or extremely enriched (+++) CO_2 conditions with (+N) or without (-N) NO_3^-.

Treatment	Alkalinity (μmol L^{-1})	pH	DIC (μmol L^{-1})	pCO_2 (μatm)
-CO_2-N	297 ± 6.06 (A)	8.38 ± 0.09 (B)	297 ± 7.17 (A)	149 ± 10.4 (A)
-CO_2+N	560 ± 64.2 (B)	9.03 ± 0.03 (C)	544 ± 6.18 (B)	61.9 ± 1.56 (A)
+++CO_2-N	479 ± 13.3 (B)	7.11 ± 0.03 (A)	624 ± 10.0 (C)	3895 ± 177 (B)
+++CO_2+N	788.18 ± 96.5 (C)	7.17 ± 0.01 (A)	956 ± 14.3 (D)	4478 ± 669 (C)

Average and standard deviation values are represented before and after "±", respectively. Letters in parentheses represent significant differences between experimental treatments (Two-way ANOVA; Tukey's HSD post-hoc; $p < 0.05$).

3.2. Effect of CO_2 and NO_3^- on Dolichospermum sp. 54 (anatoxin-producer)

During the experiment with *Dolichospermum* sp. 54, pH and pCO_2 values of decreased and increased, respectively, at higher CO_2 delivery rates (Table 2). NO_3^- availability and CO_2 enrichment exhibited a significant interactive effect (Two-way ANOVA; $p < 0.001$) on growth rates (μ_{max} = day^{-1}), for though -CO_2–N cultures exhibited significantly (Tukey's HSD; $p < 0.05$) higher growth rates (0.87 ± 0.04 day^{-1}) than -CO_2+*N* cultures (0.22 ± 0.03 day^{-1}), the growth rates of +++CO_2+*N* cultures exhibited significantly (Tukey's HSD; $p < 0.05$) higher growth (1.03 ± 0.08 day^{-1}) than those of +++CO_2–N cultures (0.25 ± 0.03 day^{-1}; Fig. 2A). +*N* cultures exhibited significantly (Tukey's HSD; $p < 0.05$) faster growth rates at each successive enriched CO_2 level relative to +*N* cultures grown under lower CO_2 conditions (Fig. 2A).

CO_2 enrichment and NO_3^- availability also exhibited a significant antagonistic effect (Two-way ANOVA; $p < 0.001$) on N_2 fixation (Fig. 2B). At each CO_2 level, -N cultures exhibited significantly (Tukey's HSD; $p < 0.05$) higher N_2 fixation rates than +*N* cultures (Fig. 2B). The N_2 fixation rates of ++CO_2+*N* (0.01 ± 0.004 pmol N_2-fixed cell^{-1} day^{-1}) and +++CO_2+*N* (0.02 ± 0.002 pmol N_2-fixed cell^{-1} day^{-1}) cultures were also significantly (Tukey's HSD; $p < 0.05$) faster than those of -CO_2+*N* cultures (0.001 ± 0.0002 pmol N_2-fixed cell^{-1} day^{-1}; Fig. 2B). The N_2 fixation rates of +++CO_2–N (0.15 ± 0.01 pmol N_2-fixed cell^{-1} day^{-1}) were significantly higher than any other treatment (Fig. 2B). As with N_2 fixation, the H:TC of *Dolichospermum* sp. 54 was significantly antagonistically affected (Two-way ANOVA; $p < 0.05$) by NO_3^- availability and CO_2 enrichment (Fig. 2C). -N cultures enriched with CO_2 exhibited significantly (Tukey's HSD; $p < 0.05$) higher H:TC (+++CO_2-N = 0.06 ± 0.005; ++CO_2–N 0.06 ± 0.003; +CO_2-N = 0.05 ± 0.003) than -CO_2–N cultures (0.04 ± 0.01), while the H:TC of +++CO_2+*N* cultures (0.01 ± 0.002) was significantly (Tukey's HSD; $p < 0.05$) higher than those of -CO_2+*N* (0.0005 ± 0.0002) and +CO_2+*N* (0.001 ± 0.0009) cultures. Moreover, N_2 fixation and H:TC were significantly positively correlated (F-test; $R^2 = 0.80$, $p < 0.001$).

ATX-A cellular quotas were significantly antagonistically affected (Two-way ANOVA; $p < 0.001$) by CO_2 enrichment and NO_3^- availability (Fig. 2D). ATX-A quotas of +++CO_2–N (0.63 ± 0.05 pg ATX-A cell^{-1}) and +CO_2–N (0.53 ± 0.05 pg ATX-A cell^{-1}) were significantly (Tukey's

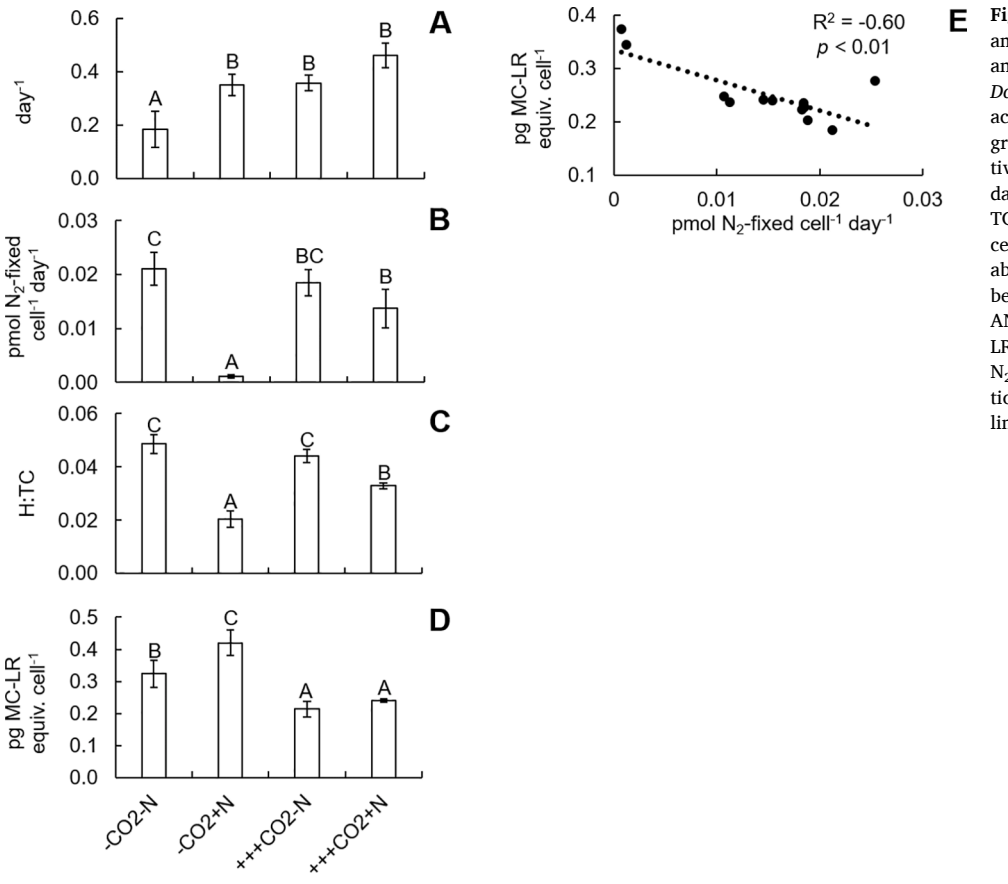

Fig. 1. Effects of NO_3^- depletion (-N) and amendment (+N) as well as ambient air (-CO_2) and air extremely (+++) enriched with CO_2 on *Dolichospermum* sp. 90. These effects are characterized specifically with respect to maximum growth rate (A; day^{-1}), N_2 fixation rates relative to cell density (B; pmol N_2-fixed cell^{-1} day^{-1}), heterocyst – to – total cell ratios (C; H:TC), and microcystin-LR (MC-LR) equivalent cell quotas (D; pg MC-LR equiv. cell^{-1}). Letters above bars represent significant differences between experimental groups (Two-way ANOVA; Tukey's HSD post-hoc; $p < 0.05$). MC-LR cell quotas were also plotted as functions of N_2 fixation rates (E). Coefficient of determination (R^2) and p - values of statistical (F) tests of linear regressions are denoted.

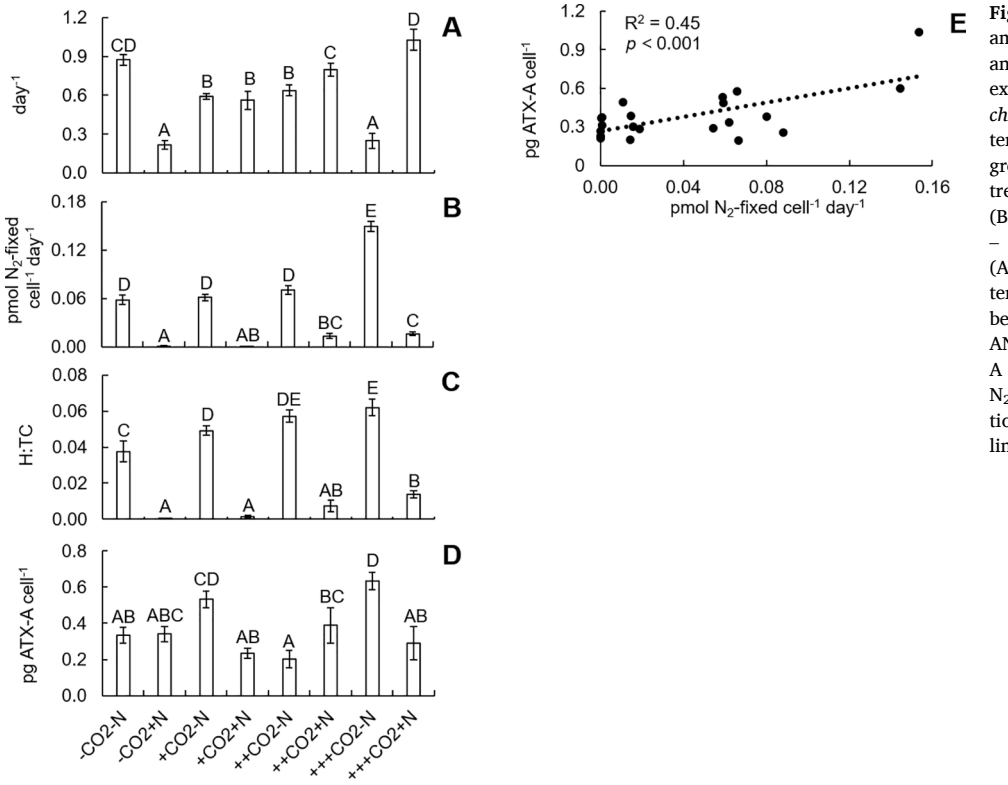

Fig. 2. Effects of NO_3^- depletion (-N) and amendment (+*N*) as well as ambient air (-CO_2) and air moderately (+), highly (++), and extremely (+++) enriched with CO_2 on *Dolichospermum* sp. 54. These effects are characterized specifically with respect to maximum growth rate (A; day^{-1}), N_2 fixation rates across treatments and relative to time and cell density (B; pmol N_2-fixed cell^{-1} day^{-1}), heterocyst – to – total cell ratios (C; H:TC), and anatoxin-a (ATX-A) cell quotas (D; pg ATX-A cell^{-1}). Letters above bars represent significant differences between experimental groups (Two-way ANOVA; Tukey's HSD post-hoc; $p < 0.05$). ATX-A cell quotas were also plotted as functions of N_2 fixation rates (E). Coefficient of determination (R^2) and p - values of statistical (F) tests of linear regressions are denoted.

Table 2
Carbonate chemistry of *Dolichospermum* sp. 54 cultures grown under ambient (-), moderately (+), highly (++), or extremely enriched (+++) CO_2 conditions with (+N) or without (-N) NO_3^-.

Treatment	Alkalinity (μmol L^{-1})	pH	DIC (μmol L^{-1})	pCO_2 (μatm)
-CO_2-N	206 ± 7.64 (A)	8.60 ± 0.03 (D)	186 ± 1.33 (A)	607 ± 0.01 (A)
-CO_2+N	425 ± 23.1 (B)	8.28 ± 0.15 (CD)	415 ± 41.1 (B)	630 ± 34.6 (A)
+CO_2-N	240 ± 41.4 (A)	8.11 ± 0.27 (CD)	288 ± 58.6 (A)	1668 ± 21.6 (B)
+CO_2+N	588 ± 4.33 (C)	8.09 ± 0.17 (C)	615 ± 3.47 (C)	1607 ± 7.21 (B)
++CO_2-N	269 ± 43.6 (A)	6.75 ± 0.06 (A)	401 ± 46.0 (B)	3507 ± 136 (C)
++CO_2+N	625 ± 15.6 (C)	7.36 ± 0.22 (B)	736 ± 21.9 (D)	2984 ± 57.7 (D)
+++CO_2-N	278 ± 4.54 (A)	6.48 ± 0.02 (A)	500 ± 10.1 (BC)	5845 ± 247 (E)
+++CO_2+N	551 ± 25.6 (C)	6.86 ± 0.22 (A)	796 ± 46.5 (D)	5805 ± 73.1 (E)

Average and standard deviation values are represented before and after "±", respectively. Letters in parentheses represent significant differences between experimental treatments (Two-way ANOVA; Tukey's HSD post-hoc; $p < 0.05$).

HSD; $p < 0.05$) higher than those of +*N* cultures grown at the same CO_2 levels (+CO_2+*N* = 0.23 ± 0.03 pg ATX-A $cell^{-1}$; +++CO_2+*N* = 0.29 ± 0.09 pg ATX-A $cell^{-1}$). Conversely, the ATX-A quotas of ++CO_2+*N* (0.39 ± 0.10 pg ATX-A $cell^{-1}$) were significantly (Tukey's HSD; $p < 0.05$) higher than those of ++CO_2–N (0.20 ± 0.05 pg ATX-A $cell^{-1}$) cultures. Finally, ATX-A cell quotas were significantly (F-test; $R^2 = 0.45$, $p < 0.001$) positively correlated with N_2 fixation rates (Fig. 2E).

3.3. Effect of CO_2 and NO_3^- on D. circinale ACBU02 (saxitoxin-producer)

During the experiment with *D. circinale* ACBU02, pH and pCO_2 values decreased and increased, respectively, at higher CO_2 delivery rates (Table 3). CO_2 enrichment and NO_3^- availability had a significant antagonistic effect (Two-way ANOVA; $p < 0.05$) on the growth rates (μ_{max} = day^{-1}) of *D. circinale* ACBU02 (Fig. 3A). Growth rates of +CO_2+*N* (0.34 ± 0.02 day^{-1}) and +++CO_2+*N* (0.38 ± 0.04 day^{-1}) cultures were significantly (Tukey's HSD; $p < 0.05$) higher relative to those of -CO_2+*N* (0.23 ± 0.03 day^{-1}; Fig. 3A).

NO_3^- availability and CO_2 enrichment exhibited a significant antagonistic effect (Two-way ANOVA; $p < 0.01$) on N_2 fixation (Fig. 3B). Consistent with the other strains, +*N* cultures exhibited significantly (Tukey's HSD; $p < 0.05$) lower N_2 fixation rates relative to -N cultures. In addition, the -CO_2–N (0.06 ± 0.01 pmol N_2-fixed $cell^{-1}$ day^{-1}) and +++CO_2–N (0.06 ± 0.01 pmol N_2-fixed $cell^{-1}$ day^{-1}) cultures exhibited significantly (Tukey's HSD; $p < 0.05$) higher N_2 fixation rates than those of any other treatment. +++CO_2+*N* cultures also exhibited significantly (Tukey's HSD; $p < 0.05$) higher N_2 fixation rates (0.03 ± 0.003 pmol N_2-fixed $cell^{-1}$ day^{-1}) than any other +*N* treatment (~0.01 ± 0.003 pmol N_2-fixed $cell^{-1}$ day^{-1}). N_2 fixation rates were significantly (F-test; $R^2 = 0.55$, $p < 0.001$) positively correlated with H:TC. In contrast to N_2 fixation, H:TC was only significantly altered by NO_3^- (Two-way ANOVA; $p < 0.001$), decreasing relative to -N cultures at every CO_2 level (Fig. 3C).

CO_2 enrichment (Two-way ANOVA; $p < 0.001$) significantly decreased total STX cellular quotas, while NO_3^- availability significantly (Two-way ANOVA; $p < 0.01$) influenced STX quotas under elevated CO_2 conditions (Fig. 3D). -N cultures enriched with CO_2 all exhibited significantly (Tukey's HSD; $p < 0.05$) lower toxin quotas (+CO_2-N = 0.09 ± 0.01 pg Total STX $cell^{-1}$; ++CO_2-N = 0.11 ± 0.01 pg Total STX $cell^{-1}$; +++CO_2-N = 0.09 ± 0.002 pg Total STX $cell^{-1}$) than -CO_2–N cultures (0.15 ± 0.01 pg Total STX $cell^{-1}$). +++CO_2+*N* cultures also exhibited significantly (Tukey's HSD; $p < 0.05$) higher toxin quotas (0.12 ± 0.002 pg Total STX $cell^{-1}$) than +++CO_2–N (0.09 ± 0.003 pg Total STX $cell^{-1}$) cultures. Saxitoxin congeners dcGTX2, dcGTX3, STX, GTX1, and GTX3 exhibited a similar trend at the extremely enriched CO_2 level, with the average quota of each congener in +++CO_2+*N* cultures being significantly (Tukey's HSD; $p < 0.05$) higher than those of +++CO_2–N cultures (Supplemental Fig. 2B). These differences reflect the significant (Two-way ANOVA; $p < 0.05$) antagonistic effect NO_3^- availability and CO_2 enrichment had on the quotas of each of these saxitoxin congeners (Supplemental Table 2). There was also a marginally significant negative correlation between STX quotas and N_2 fixation rates (F-test; $R^2 = -0.20$, $p < 0.10$, Fig. 3E).

Table 3
Carbonate chemistry of *D. circinale* ACBU02 cultures grown under ambient (-), moderately (+), highly (++), or extremely enriched (+++) CO_2 conditions with (+N) or without (-N) NO_3^-.

Treatment	Alkalinity (μmol L^{-1})	pH	DIC (μmol L^{-1})	pCO_2 (μatm)
-CO_2-N	263 ± 6.97 (A)	8.48 ± 0.04 (F)	252 ± 20.4 (A)	254 ± 4.33 (A)
-CO_2+N	516 ± 94.1 (B)	8.52 ± 0.05 (F)	500 ± 86.5 (B)	289 ± 8.72 (A)
+CO_2-N	318 ± 13.3 (A)	7.15 ± 0.06 (D)	383 ± 18.3 (AB)	1741 ± 326 (B)
+CO_2+N	717 ± 79.9 (C)	7.91 ± 0.02 (E)	743 ± 79.2 (CD)	896 ± 12.1 (A)
++CO_2-N	272 ± 9.70 (A)	6.46 ± 0.04 (B)	499 ± 12.5 (B)	5784 ± 292 (C)
++CO_2+N	634 ± 113 (BC)	6.84 ± 0.06 (C)	845 ± 117 (D)	5605 ± 77.7 (C)
+++CO_2-N	199 ± 8.80 (A)	6.13 ± 0.03 (A)	544 ± 24.4 (BC)	8759 ± 317 (D)
+++CO_2+N	506 ± 78.4 (B)	6.49 ± 0.02 (B)	885 ± 121 (D)	10,606 ± 121 (E)

Average and standard deviation values are represented before and after "±", respectively. Letters in parentheses represent significant differences between experimental treatments (Two-way ANOVA; Tukey's HSD post-hoc; $p < 0.05$).

4. Discussion

This study explored how differing levels of N and CO_2, reflective of some current environmental conditions as well as of future carbon emission scenarios, affected growth, toxin production, and N_2 fixation by the CHAB, *Dolichospermum*. Findings largely supported the first hypothesis as growth and N_2 fixation rates were significantly enhanced under CO_2 enrichment, though whether rates of both physiological processes were faster was largely dependent on NO_3^- levels. Consistent with the second hypothesis, the cellular quotas of N-rich cyanotoxins (MC and STX) were significantly higher and lower in response to NO_3^- amendment and CO_2 enrichment, respectively, while quotas of the N-poor cyanotoxin ATX-A were significantly higher when NO_3^--deprived cultures were enriched with CO_2. Lastly, N_2 fixation was generally enhanced under CO_2 enrichment and suppressed by NO_3^- with CO_2 and NO_3^- interacting antagonistically as N_2 fixation was elevated when NO_3^--enriched *Dolichospermum* spp. cultures were grown under elevated CO_2 levels compared to cultures grown under lower CO_2 conditions. Collectively, these findings provide important new insights regarding the manner which CO_2 and NO_3^- can act and interact to control the growth, nitrogen fixation, and cellular quotas of microcystin, anatoxin, and saxitoxin in *Dolichospermum* spp.

4.1. Elevated CO_2 levels and nitrate enrichment enhance Dolichospermum spp. growth rates

Despite the ability of Nostocales to perform N_2 fixation under fixed N-deplete conditions, the availability of fixed N has been shown to promote the growth and/or dominance of several genera in this order

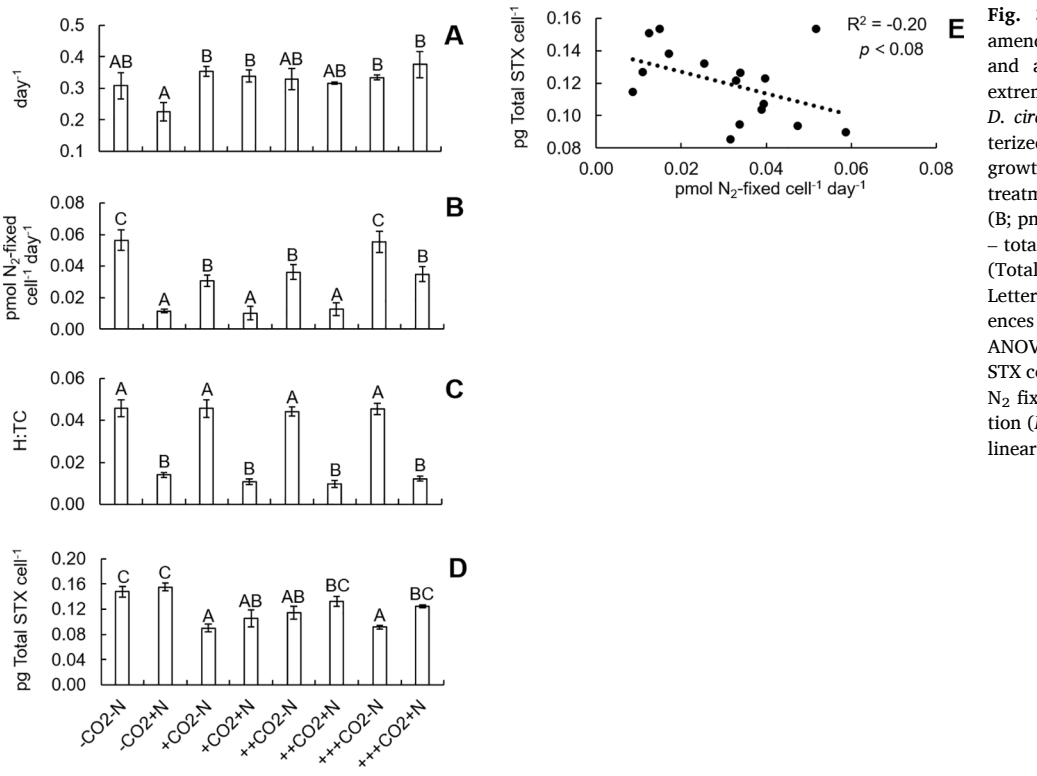

Fig. 3. Effects of NO_3^- depletion (-N) and amendment (+N) as well as ambient air (-CO_2) and air moderately (+), highly (++), and extremely (+++) enriched with CO_2 on *D. circinale* ACBU02. These effects are characterized specifically with respect to maximum growth rate (A; day^{-1}), N_2 fixation rates across treatments and relative to time and cell density (B; pmol N_2-fixed $cell^{-1}$ day^{-1}), heterocyst – to – total cell ratios (C; H:TC), and total saxitoxin (Total STX) cell quotas (D; pg Total STX $cell^{-1}$). Letters above bars represent significant differences between experimental groups (Two-way ANOVA; Tukey's HSD post-hoc; $p < 0.05$). Total STX cell quotas were also plotted as functions of N_2 fixation rates (E). Coefficient of determination (R^2) and p - values of statistical (F) tests of linear regressions are denoted.

(Dolman et al., 2012; Zulkefli and Hwang, 2020). Here, NO_3^- amendment had a positive effect on *Dolichospermum* spp. growth, although this effect was only significant under ambient CO_2 conditions for *Dolichospermum* sp. 90. In contrast, cultures of *Dolichospermum* sp. 54 and *D. circinale* ACBU02 amended with NO_3^- exhibited significantly enhanced growth only when CO_2 levels were elevated. This effect was most obvious for *Dolichospermum* sp. 54, NO_3^--deprived cultures of which exhibited markedly lower growth rates at each successively enriched CO_2 level, while the reverse was true for cultures amended with NO_3^-. Consequently, this led to a complete reversal of the effect of NO_3^- on *Dolichospermum* sp. 54 growth, with +++CO_2+*N* cultures not only exhibiting significantly faster growth rates than +++CO_2–N cultures, but significantly faster growth rates than any other treatment in the experiment aside from those of -CO_2–N cultures. Similarly, +++CO_2+*N* cultures of *D. circinale* ACBU02 and *Dolichospermum* sp. 90 also exhibited, respectively, significantly and non-significantly higher growth rates than those of -CO_2+N cultures. This finding, coupled with the equal (sp. 90) and greater (sp. 54 and ACBU02) statistical significance of CO_2 as a treatment factor for growth rates relative to NO_3^-, suggests that CO_2 enrichment played a more significant role than NO_3^- in promoting *Dolichospermum* growth rates. Furthermore, CO_2 and NO_3^- -enriched conditions may yield the highest biomass of these and other CHAB-forming taxa. This could have been facilitated by the down-regulation of the carbon concentrating mechanism (CCM) due CO_2 enrichment (Woodger et al., 2003) and the upregulation of genes associated with photosynthesis and transporters involved in C and N uptake (Mehta et al., 2019).

Cyanobacteria and other phytoplankton can exhibit DIC-limitation in eutrophic freshwater systems (Hein, 1997; Jannson et al., 2012; Symes and van Ogtrop, 2019). While some eutrophic lakes exhibit CO_2 supersaturation, with pCO_2 levels in extreme cases ranging from 5000 - 83, 700 μatm, 44 - 60% of samples collected from North and South American lentic systems during the summer experienced CO_2 undersaturation relative to the atmosphere (Balmer and Downing, 2011; Zagarese et al., 2021). Summer CHAB communities can also exhibit significantly higher net primary production rates in response to increases in CO_2 and lake alkalinity (Shi et al., 2017). Experiments with *Dolichospermum* spp. described in this study were consistent with these findings, as +++CO_2+N cultures exhibited significantly elevated DIC (> 740 μmol L^{-1}) and alkalinity (> 420 μmol L^{-1}) levels, as well as enhanced growth rates, relative to those of -CO_2–N cultures (DIC and alkalinity < 310 μmol L^{-1}). The higher alkalinities of CO_2-enriched +N cultures further reflects how NO_3^- assimilation by phytoplankton can increase lake alkalinity by consuming H^+ ions, subsequently increasing the storage capacity of HCO_3^- in freshwater systems (Schindler et al., 1985; Verspagen et al., 2014). Collectively, this suggests natural communities of *Dolichospermum* will exhibit more intense blooms in eutrophic, specifically NO_3^--rich, CO_2-enriched freshwater systems.

The effect of fixed N on cyanobacterial communities can be influenced by the form of N used for growth. While NO_3^- is usually the dominant form of fixed inorganic N in freshwater ecosystems (Durand et al., 2011; Hessen, 2013), NO_3^- reduction is more energetically demanding than NH_4^+ assimilation or urea hydrolysis (Sanz et al., 1995; Harke and Gobler, 2015; Herrero and Flores, 2018), and growth on reduced nitrogen compounds is often significantly faster than when *Dolichospermum* spp. are grown on NO_3^- (Rhee and Lederman, 1983; Qian et al., 2017). This may partly explain why -CO_2+*N* cultures of *Dolichospermum* sp. 54 and *D. circinale* ACBU02 exhibited significantly and non-significantly reduced growth rates relative to -CO_2–N cultures, respectively, as for these strains diazotrophy was seemingly preferable to NO_3^- assimilation at ambient CO_2 levels (Meeks et al., 1983). The inability of nitrate to promote significantly higher growth rates in *D. circinale* ACBU02 relative to -N cultures at all CO_2 levels may also reflect the strain's origin from a reservoir in the subtropics (Pereyra et al., 2017), where NO_3^- levels are particularly low due to a lack of seasonal ice cover (Powers et al., 2017; Maberly et al., 2020). This was not the case for *Dolichospermum* sp. 90 cultures, however, which exhibited significantly faster growth rates when grown with -CO_2+N relative to -CO_2–N. Diazotrophy can also be preferable to NO_3^- assimilation for the growth of other *Dolichospermum* taxa at elevated CO_2, even when millimolar concentrations of nitrate (Kang et al., 2005) are available. However, this was likely not the case for *Dolichospermum* sp.

54 cultured under $+++CO_2$+*N* conditions, as they exhibited significantly higher growth rates than those of any other +N treatment. That NO_3^- amendment under $+++CO_2$ conditions led to significantly (*D. circinale* ACBU02 and sp. 54) and non-significantly (sp. 90) higher growth rates relative to those of -CO2+N cultures suggests that *Dolichospermum* may prefer NO_3^- more under elevated CO_2 conditions in freshwater ecosystems, perhaps due to an increased N demand brought on by higher DIC levels.

4.2. CO_2 enrichment enhances N_2 fixation rates in Dolichospermum spp

In all experiments, elevated CO_2 levels promoted enhanced N_2 fixation rates in NO_3^-- amended cultures relative to cultures also amended with NO_3^- but grown at ambient CO_2 levels, indicating that freshwater CO_2 enrichment may enhance diazotrophy in eutrophic ecosystems. Levels of CO_2 used in this study were reflective of both current conditions in differing lakes, as well as some future climate change scenarios with ambient conditions (-N-CO_2) likely representative of current conditions in oligotrophic, lower CO_2 lakes (Cole et al., 1994; Balmer and Downing, 2011; Hasler et al., 2016). Enhanced N_2 fixation under elevated CO_2 conditions has been reported previously in both marine (Levitan et al., 2010; Hutchins et al., 2015; Rees et al., 2017) and freshwater (Wannicke et al., 2012; Wannicke et al., 2021) cyanobacteria. Results presented here also indicate that nitrogenase activity and NO_3^- assimilation were used simultaneously to meet nitrogen demands, which has also been reported in other *Dolichospermum* taxa under ambient (Elder and Parker, 1984; Sanz et al., 1995) and elevated (Kang et al., 2005) CO_2 conditions. *Dolichospermum* sp. 54 cultures grown under $+++CO_2$–N conditions also exhibited significantly higher N_2 fixation rates than any other treatment, and further exhibited significantly reduced growth relative to NO_3^--deprived cultures grown under ambient or less enriched CO_2 levels. This suggests that enhanced diazotrophy alone will be insufficient for some cyanobacteria to meet fixed N demands for optimal growth in acidified freshwater systems with low fixed nitrogen-to-phosphorus (N:P) ratios.

4.3. CO_2 enrichment and nitrogen availability regulate concentrations of microcystin, anatoxin, and saxitoxin in Dolichospermum spp

CO_2-enrichment in NO_3^--deprived cultures caused significant reductions in cellular MC and STX concentrations in *Dolichospermum* sp. 90 and *D. circinale* ABU02, respectively, relative to NO_3^--amended and NO_3^--deprived cultures grown under ambient CO_2 conditions. In contrast, NO_3^--amended cultures of both strains exhibited non-significantly (sp. 90) and significantly (*D. circinale* ACBU02) higher cellular toxin quotas relative to NO_3^--deprived cultures grown under CO_2-enriched ($+++CO_2$) conditions. Elevated quotas of N-rich cyanotoxins such as certain microcystin and saxitoxin congeners can occur when cyanobacteria are grown under fixed N-replete conditions (Van de Waal et al., 2009; Van de Waal et al., 2014), although this trend is largely limited to observations of microcystin-producing, non-diazotrophic cyanobacteria (Downing et al., 2005; Davis et al., 2015; Harke and Gobler, 2015) and has been observed in response to only changing fixed N supply (Van de Waal et al., 2014). Non-diazotrophic cyanobacteria also exhibit reduced microcystin quotas under fixed N-deplete, elevated CO_2 conditions (Van de Waal et al., 2011; Liu et al., 2016). While this is consistent with findings presented in this study, it is important to note that the growth rates and MC quotas of *Dolichospermum* sp. 90 were significantly negatively correlated, suggesting that diazotrophic cyanobacteria such as *Dolichospermum* prioritize the use of NO_3^- for growth over the production of N-rich cyanotoxins under CO_2 enrichment. Similar results have been reported in *Nodularia*, which exhibits enhanced growth and decreased quotas of the N-rich toxin nodularin under CO_2 enrichment and NO_3^--replete conditions (Wannicke et al., 2021). Collectively, findings suggest that blooms of Nostocales taxa may produce significantly less N-rich cyanotoxins relative to their enhanced biomass under CO_2 enrichment.

How environmental factors alter cyanobacterial STX quotas and/or production is poorly understood (Neilan et al., 2013; Visser et al., 2016; Christensen and Kahn, 2020). This study is the first to characterize the effects of elevated CO_2 and fixed N availability on *Dolichospermum* STX quotas. In studies where CO_2 levels were not manipulated, cellular STX concentrations in other Nostocales isolates were not significantly increased with increasing concentrations of NO_3^- (Velzeboer et al., 2001; Yunes et al., 2009; Cirés et al., 2017). Saxitoxin production in *Raphidiopsis raciborskii*, formerly *Cylindrospermopsis raciborskii* (Aguilera et al., 2018), was shown to increase at high total nitrogen-to-phosphorus ratios caused by high NO_3^- (Chislock et al., 2014), and significantly decrease under elevated CO_2 levels (Vilar and Molica, 2020). During the present study, CO_2 enrichment caused significantly lower cellular total saxitoxin quotas, yet $+++CO_2$+*N* cultures exhibited significantly higher saxitoxin quotas relative to $+++CO_2$–N cultures. This demonstrates the antagonistic nature of NO_3^- and CO_2 in driving STX quotas significantly higher and lower, respectively, under extremely enriched CO_2 conditions. Cellular quotas of several saxitoxin congeners, such as non-sulfated STX, the gonyautoxins GTX1 and GTX3, and the decarbamoyl-gonyautoxins dcGTX2 and dcGTX3 (Wiese et al., 2010), which are the most toxic paralytic shellfish poisons (PSPs) produced by phytoplankton (Genenah and Shimizu, 1981; Sullivan et al., 1985; Selwood et al., 2017), were also significantly higher in $+++CO_2$+N cultures than in $+++CO_2$–N cultures. Collectively, this indicates that as CO_2 levels in freshwater ecosystems rise, the importance of NO_3^- availability in enhancing saxitoxin production, particularly the production of highly toxic congeners, will increase. Subsequently, this will increase risks to human and animal health.

As with STX, the effects of environmental conditions on ATX-A production in cyanobacteria is also poorly understood (Neilan et al., 2013; Visser et al., 2016; Christensen and Kahn, 2020). This is the first study to observe the effects of CO_2 enrichment and NO_3^- availability on *Dolichospermum* ATX-A quotas. When grown under extremely enriched levels of CO_2 and deprived of NO_3^-, ATX-A quotas of *Dolichsopermum* sp. 54 were significantly higher than those of NO_3^--amended cultures grown at the same CO_2 level. These data, coupled with N_2 fixation rates and ATX-A cellular quotas being negatively correlated with growth rates, and ATX-A quotas being positively correlated with N_2 fixation rates, indicates that *Dolichsopermum* sp. 54 anatoxin synthesis is enhanced under C-replete as well as fixed N and P-deplete conditions (Gagnon and Pick, 2012), and may serve as an anti-grazing (Toporowska et al., 2014) or allelopathic (Chia et al., 2019) strategy when cells are in the stationary phase of growth (Harland et al., 2013; Heath et al., 2016). Moreover, this suggests that Nostocales cell quotas of cyanotoxins with a high C:N ratio such as ATX-A (Van de Waal et al., 2014) will increase in CO_2-enriched freshwater systems.

N_2 fixation was significantly negatively correlated with MC and STX quotas and significantly positively correlated with ATX-A quotas. This is the first study to report significant linear relationships between the N_2 fixation rates and cyanotoxin quotas of *Dolichospermum*. The mechanism by which N_2 fixation may influence cyanotoxin production and the expression of genes encoding for these toxins is unknown. Given that cyanotoxins are generally regarded as secondary metabolites (Carmichael, 1992; O'Neil et al., 2012), declines in MC and STX cellular quotas concurrent with increases in N_2 fixation are likely representative of *Dolichospermum* spp. prioritizing growth and C:N homeostasis over toxin synthesis. Conversely, an increase in ATX-A quota and N_2 fixation suggests that the synthesis of this relatively N-poor cyanotoxin is not energetically costly for *Dolichospermum* spp. under CO_2 enrichment. In fact, enhanced ATX-A production may provide intracellular stoichiometric balance under CO_2-enriched, fixed N-deplete conditions. Indeed, a recent study reported a significant increase in anatoxin cellular quotas in response to low fixed N and low P that coincided with the significant upregulation of genes belonging to the anatoxin synthesis (*ana*) synthetase gene cluster (Kramer et al., 2022). Future studies should

determine whether cellular cyanotoxin quotas and the expression of genes associated with toxin synthesis are similarly affected in response to CO_2 enrichment and fixed N availability.

4.4. Ecosystem implications

Enhanced atmospheric CO_2 uptake in freshwater systems and its effect on cyanobacteria have only recently become a focal point of research (Ma and Wang, 2021). Eutrophic systems are especially at risk of CO_2 enrichment, as enhanced CHAB growth can result in the depletion of inorganic carbon stocks and subsequently lead to increased uptake of atmospheric CO_2 (Balmer and Downing, 2011; Zagarese et al., 2021). The Laurentian Great Lakes, for instance, which constitute the largest freshwater system on Earth that regularly experiences seasonal CHABs (McKindles et al., 2020), are expected to exhibit a decline in mean pH ranging from 0.29 to 0.46 units by the end of the 21st century (Phillips et al., 2015). The findings of this study suggest that N_2 fixation and cyanobacterial productivity in eutrophic freshwater ecosystems undergoing progressive acidification may increase in the future, with diazotrophs such as *Dolichospermum* playing a more significant role in nitrogen and carbon cycling. Enhanced N_2 fixation under high CO_2, high NO_3^- conditions could accelerate eutrophication by providing more fixed N into such ecosystems (Fig. 4). This could lead to non-N_2 fixing cyanobacteria such as *Microcystis* being promoted by fixed N provided by Nostocales taxa to initiate blooms (Beversdorf et al., 2013; Eldridge et al., 2013), as well as changes in the amounts of organic carbon and nitrogen available to higher trophic levels (Karlson et al., 2015; Adam et al., 2016).

The geographic ranges of Nostocales taxa such as *Dolichospermum* (Salmaso et al., 2015; Li et al., 2016; Capelli et al., 2017), *Aphanizomenon* (Budzyńska et al., 2019), and *Raphidiopsis* (Briand et al., 2004; Sukenik et al., 2012; Antunes et al., 2015) have expanded considerably in the past few decades, specifically towards higher latitudes. This trend has generally been attributed to rising temperatures under which cyanobacteria generally outgrow eukaryotic phytoplankton (Robarts and Zohary, 1987; Schabhüttl et al., 2013; Nalley et al., 2018). The findings presented in this study indicate that blooms of Nostocales taxa such as *Dolichospermum* may also be benefiting from progressively rising CO_2 levels. While quotas of N-rich cyanotoxins of diazotrophic cyanobacteria may decline under freshwater acidification (Fig. 4), increased growth rates due to elevated CO_2 levels, and therefore more intense blooms, may cause toxin concentrations in freshwater ecosystems to remain a significant risk to human and animal health, especially under fixed N-replete conditions.

5. Conclusions

CO_2 enrichment significantly increases the growth and N_2 fixation rates of *Dolichospermum* spp. amended with fixed N nitrogen (Fig. 4). Quotas of N-rich cyanotoxins MC and STX were significantly enhanced by NO_3^- under ambient and elevated CO_2 conditions; quotas of both toxins were suppressed by high CO_2 levels (Fig. 4). Microcystin and saxitoxin quotas significantly decreased as N_2 fixation increased, whereas ATX-A quotas increased (Fig. 4), suggesting that reliance on diazotrophy minimizes the amount of fixed N available for N-rich toxin synthesis, but is associated with enhanced production of N-poor toxins. CO_2 enrichment and NO_3^- interacted antagonistically to yield significantly higher N_2 fixation rates in NO_3^--amended cultures exposed to extremely enriched CO_2 levels relative to NO_3^--amended cultures grown at ambient CO_2 levels. This interaction could further promote

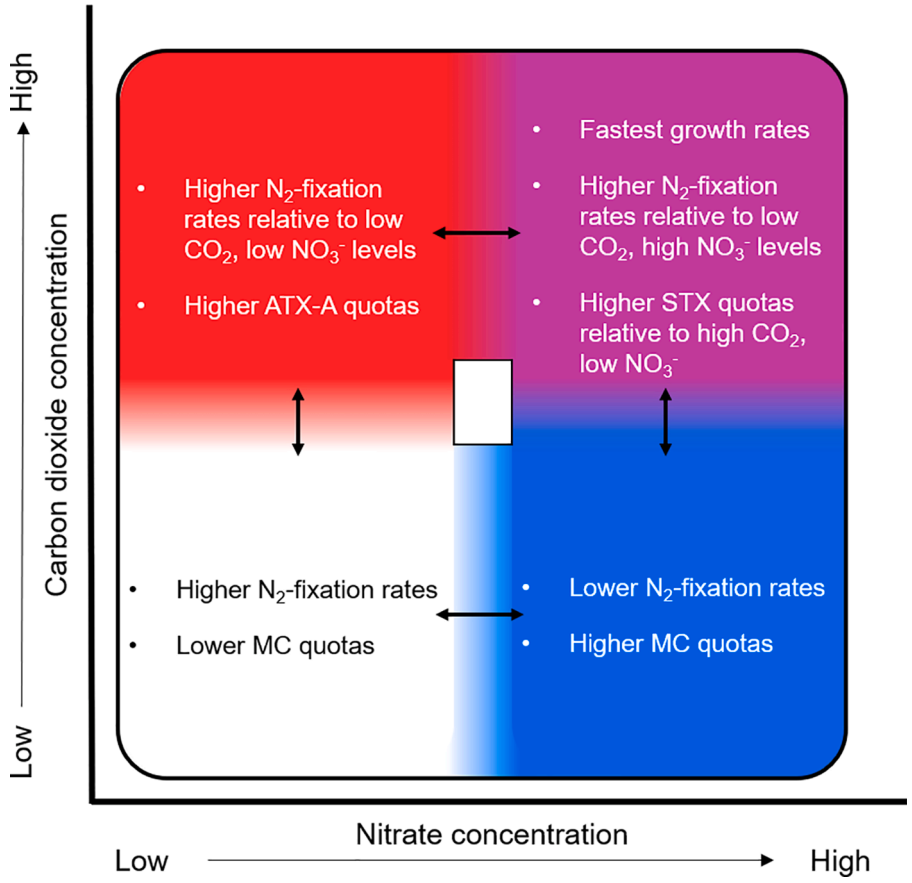

Fig. 4. The response of *Dolichospermum* to high and low carbon dioxide (CO_2) and nitrate (NO_3^-) levels with respect to growth and N_2 fixation rates as well as microcystin (MC), saxitoxin (STX), anatoxin (ATX-A) quotas.

eutrophication by providing more fixed N into such ecosystems via positive feedback. Future studies should consider how Nostocales taxa respond to different N species under elevated pCO_2 conditions and consider how toxin synthesis and C and N metabolism of diazotrophic cyanobacteria is regulated under acidification. Furthermore, the addition of other environmental factors, namely temperature, in experimental designs would be essential for better characterizing how CHAB - forming diazotrophs respond to climate change and eutrophication.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2022.102354.

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