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## EDITED BY

Rachel Ann Foster,  
Stockholm University, Sweden

## REVIEWED BY

Gregory L. Boyer,  
SUNY College of Environmental  
Science and Forestry, United States  
Yuuhiiko Tanabe,  
National Institute for Environmental  
Studies (NIES), Japan

## \*CORRESPONDENCE

Christopher J. Gobler  
christopher.gobler@stonybrook.edu

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# Nitrogen and phosphorus significantly alter growth, nitrogen fixation, anatoxin-a content, and the transcriptome of the bloom-forming cyanobacterium, *Dolichospermum*

Benjamin J. Kramer<sup>1</sup>, Jennifer G. Jankowiak<sup>2</sup>,  
Deepak Nanjappa<sup>1</sup>, Matthew J. Harke<sup>3</sup> and  
Christopher J. Gobler<sup>1\*</sup>

<sup>1</sup>School of Marine and Atmospheric Sciences, Stony Brook University, Southampton, NY, United States, <sup>2</sup>Jonah Ventures, Boulder, CO, United States, <sup>3</sup>Gloucester Marine Genomics Institute, Gloucester, MA, United States

While freshwater cyanobacteria are traditionally thought to be limited by the availability of phosphorus (P), fixed nitrogen (N) supply can promote the growth and/or toxin production of some genera. This study characterizes how growth on N<sub>2</sub> (control), nitrate (NO<sub>3</sub><sup>-</sup>), ammonium (NH<sub>4</sub><sup>+</sup>), and urea as well as P limitation altered the growth, toxin production, N<sub>2</sub> fixation, and gene expression of an anatoxin-a (ATX-A) – producing strain of *Dolichospermum* sp. 54. The transcriptomes of fixed N and P-limited cultures differed significantly from those of fixed N-deplete, P-replete (control) cultures, while the transcriptomes of P-replete cultures amended with either NH<sub>4</sub><sup>+</sup> or NO<sub>3</sub><sup>-</sup> were not significantly different relative to those of the control. Growth rates of *Dolichospermum* (sp. 54) were significantly higher when grown on fixed N relative to without fixed N; growth on NH<sub>4</sub><sup>+</sup> was also significantly greater than growth on NO<sub>3</sub><sup>-</sup>. NH<sub>4</sub><sup>+</sup> and urea significantly lowered N<sub>2</sub> fixation and *nifD* gene transcript abundance relative to the control while cultures amended with NO<sub>3</sub><sup>-</sup> exhibited N<sub>2</sub> fixation and *nifD* gene transcript abundance that was not different from the control. Cultures grown on NH<sub>4</sub><sup>+</sup> exhibited the lowest ATX-A content per cell and lower transcript abundance of genes associated ATX-A synthesis (*ana*), while the abundance of transcripts of several *ana* genes were highest under fixed N and P - limited conditions. The significant negative correlation between growth rate and cellular anatoxin quota as well as the significantly higher number of transcripts of *ana* genes in cultures deprived of

fixed N and P relative to P-replete cultures amended with  $\text{NH}_4^+$  suggests ATX-A was being actively synthesized under P limitation. Collectively, these findings indicate that management strategies that do not regulate fixed N loading will leave eutrophic water bodies vulnerable to more intense and toxic (due to increased biomass) blooms of *Dolichospermum*.

#### KEYWORDS

nitrogen, ammonium, nitrate, urea, phosphorus,  $\text{N}_2$  fixation, *Dolichospermum*, anatoxin-a

## Introduction

*Dolichospermum*, formerly known as *Anabaena* (Wacklin et al., 2009), is a cyanobacterial genus that includes toxin-producing taxa which can form harmful blooms (CHABs) in fresh (O'Neil et al., 2012; Li et al., 2016) and brackish water (Paul et al., 2016; Olofsson et al., 2020) ecosystems. Blooms of this genus are increasing in frequency, intensity, and geographic range due to climate change and eutrophication (O'Neil et al., 2012; Salmaso et al., 2015; Li et al., 2016). This genus belongs to the order Nostocales (Fogg, 1942; Issa et al., 2014), a group of dinitrogen ( $\text{N}_2$ ) - fixing cyanobacteria that develops heterocysts when fixed N is limiting (Mickelson et al., 1967; El-Shehawy and Kleiner, 2003) and utilizes the enzyme nitrogenase to convert  $\text{N}_2$  to ammonia (Wolk et al., 1994; Hoffman et al., 2014).

Primary productivity in freshwater ecosystems is traditionally considered phosphorus (P) - limited (Correll, 1999; Blomqvist et al., 2004) because  $\text{N}_2$ -fixing cyanobacteria, or diazotrophs, which bloom under P-replete conditions (Gobler et al., 2016; Wang et al., 2021), produce newly fixed N to support phytoplankton growth (Smith, 1983, 2016; Schindler et al., 2008). The proportion of newly fixed N relative to total nitrogen in eutrophic lakes, however, can be extremely variable (6–80%), and is often regarded as insufficient to supply CHAB taxa with N sufficient to achieve optimal biomass (Howarth et al., 1988; Scott and McCarthy, 2010; Hellweger et al., 2016) due to micronutrient and light limitation (Wurtsbaugh and Horne, 1983; Lewis and Levine, 1984) as well as denitrification (Paerl, 2017). *Dolichospermum* populations do not necessarily bloom in response to P-enrichment (Paerl et al., 2014), and in some cases exhibit a higher demand for P than non-diazotrophic cyanobacteria (Wan et al., 2019). Collectively, these data suggest that P-enrichment alone is insufficient for *Dolichospermum* to achieve maximum growth rates, and that diazotrophy may not fully meet the fixed N demands required for Nostocales to bloom (Dolman et al., 2012; Shatwell and Köhler, 2018; Chaffin et al., 2019; Gamez et al., 2019). Furthermore, the fixed N species preferred by *Dolichospermum* to achieve optimal growth rates and how members of this genus respond to fixed

N and P- depletion are poorly understood and warrant further investigation.

Nostocales also significantly reduce nitrogenase activity and upregulate metabolic pathways for fixed N assimilation when certain fixed N compounds are abundant, though the degree of reduced  $\text{N}_2$  fixation is dependent on the fixed N species provided and not always associated with enhanced growth (Sanz et al., 1995; Mekonnen et al., 2002; Zulkefli and Hwang, 2020). The effects of fixed N deprivation on *Dolichospermum* genome expression have been studied, with genes associated with heterocyst development and  $\text{N}_2$  fixation being significantly upregulated, for instance (Ehira et al., 2003; Flaherty et al., 2011; He et al., 2021). However, studies that have described how the *Dolichospermum* genome responds to different fixed N species ( $\text{NH}_4^+$ ,  $\text{NO}_3^-$ ) have never included urea, which can significantly suppress  $\text{N}_2$  fixation and enhance growth rates and toxin production (Ge et al., 1990; Qian et al., 2017). Since the 1980s, urea has also become the predominant fixed N fertilizer in the United States (Paerl et al., 2016), and its use in China is significantly positively correlated with the duration and number of CHABs (Glibert et al., 2014). The effects of P depletion on *Dolichospermum* genome expression availability (Teikari et al., 2015) have also never been coupled to changes in fixed N availability and would be essential for understanding how co-limitation affects this genus.

*Dolichospermum* spp. can produce a wide variety of toxins, including the neurotoxic congener anatoxin-a (ATX-A) (Devlin et al., 1977; Sanchez et al., 2014; Christensen and Kahn, 2020). ATX-A mimics the action of the neurotransmitter acetylcholine (Carmichael et al., 1979; Spivak et al., 1980), causing muscle fatigue, paralysis, or even death (Carmichael, 1994; Humpage et al., 1994; Lilleheil et al., 1997), and is synthesized by enzymes encoded by genes belonging to the anatoxin synthetase gene (*ana*) cluster (Rantala-Ylinen et al., 2011). ATX-A - producing *Dolichospermum* strains originate from several continents, including North America, Europe, and Australia (John et al., 2019; Österholm et al., 2020). While ATX-A concentrations and the growth of some Nostocales strains can significantly increase in response to fixed N enrichment (Dolman et al., 2012; Qian et al., 2017) and a higher dissolved N:P ratio

(Toporowska et al., 2016), other strains exhibit significantly reduced ATX-A quotas but significantly higher biomass in response to  $\text{NO}_3^-$  amendment (Gagnon and Pick, 2012). Thus, the effects of different fixed N forms and P-limitation on *Dolichospermum* ATX-A production are unclear and warrant further investigation.

Ultimately, while *Dolichospermum* growth and  $\text{N}_2$  fixation rates can be responsive to fixed N, the differential effects of different fixed N species as well as fixed N and P co-limitation on members of this genus have not been investigated. Thus, the objective of this study was to quantify the effects of  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , and urea, as well as fixed N and P co-limitation, on the growth,  $\text{N}_2$  fixation, photosystem II photosynthetic efficiency ( $F_v/F_m$ ), toxin production, and genome-wide expression of *Dolichospermum* sp. 54, an ATX-A-producing taxon. We hypothesized that the availability of any of the three fixed N forms would lead to significant reductions in  $\text{N}_2$  fixation and increases in growth, while ATX-A cellular quotas would be directly controlled by the growth rates of *Dolichospermum*. These findings will likely be useful for predicting how CHABs dominated by *Dolichospermum* and ATX-A production respond to differing nutrient regimes.

## Materials and methods

### Experimental design

*Dolichospermum* sp. strain 54 (ATX-A producer) is a non-axenic isolate from a southern Finnish lake (Rouhiainen et al., 1995) that was grown in batch cultures in the freshwater medium BG11 (Stainer et al., 1971) without fixed N (BG11-N) for several weeks prior to experimentation to promote  $\text{N}_2$  fixation. Immediately prior to experimentation, cells from these cultures, which were in early stationary phase, were enumerated (see below) and centrifuged at 3000 RCF for 10 min, the supernatant removed, and cell pellets resuspended in BG11-N to avoid changing the target fixed N concentrations in experimental treatments. 500 mL Erlenmeyer flasks containing 300 mL BG11 modified with different forms and concentrations of fixed N or without P were then inoculated with the concentrated *Dolichospermum* sp. 54 sample, and used for five experimental nutrient treatments, each with three replicates ( $n = 3$ ) having an initial culture concentration of approximately  $5 \times 10^5$  cells  $\text{mL}^{-1}$ . Target cell densities were determined from the concentrated sample ( $C_1V_1 = C_2V_2$ ), and fixed N was added post-inoculation. The five experimental treatments were: BG11 with ammonium chloride ( $\text{NH}_4^+ + \text{P} = 100 \mu\text{M}$ ), urea (urea + P = 50  $\mu\text{M}$ ), or  $\text{NaNO}_3$  ( $\text{NO}_3^- + \text{P} = 100 \mu\text{M}$ ) as nitrogen forms, BG11-N (-N + P, or the control), and BG11 without fixed nitrogen or phosphorus (-N-P). Potassium hydrogen phosphate concentrations ( $\text{K}_2\text{HPO}_4$ ) were kept the same in all treatments receiving P (0.23 mM). Flasks were then

placed in an incubator set at 21°C on a 14:10 light/dark cycle at  $\sim 40 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  and bubbled with ambient air passed through 0.2  $\mu\text{m}$  HEPA filters at a rate of  $\sim 200 \text{ mL min}^{-1}$ . Sample processing and culture maintenance began in the late morning (10:00 – 11:00) during the experiment. Cultures treated with fixed nitrogen were amended again with the same nitrogen species at the same starting concentration on days 3 through 6 of the 9-day experiment to ensure that the fixed N pool did not become depleted. While the starting dissolved N:P ratios of these treatments were less than that of Redfield (1958) in fixed N-amended treatments, concentrations of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  were kept the same to assure comparability between treatments and were kept relatively low ( $\mu\text{M}$ ), as both  $\text{NH}_4^+$  (Dai et al., 2008) and urea (Sakamoto et al., 1998) are toxic to cyanobacteria at high (mM) concentrations. Every day and prior to the final time point (day 9), 10 mL of sample was removed from each flask for cell density enumeration (5 mL) and for measuring photosynthetic efficiency (5 mL); an extra 6 mL was used to measure  $\text{N}_2$  fixation (5 mL) and alkaline phosphatase activity (1 mL). The experiment concluded after cultures had been in the exponential phase of growth for several days, differences in cell densities between treatments were apparent (Supplementary Figure 1), and -N-P cultures displayed signs of P-limitation (i.e., elevated alkaline phosphatase activity relative to the control and lower  $F_v/F_m$  relative to fixed N - amended cultures; see below).

### Culture analyses

Cells were preserved in Lugol's iodine each day then enumerated under an inverted Nikon Eclipse TS100 microscope using a gridded 1  $\text{mm}^2$  Sedgewick Rafter counting chamber. Cell densities, which exhibited exponential growth as the experiment progressed (Supplementary Figure 1), were then used to determine maximum growth rates ( $\mu_{\text{max}}$ ), measured as  $\text{day}^{-1} = (\text{LN}(N/N_0))/(t - t_0)$ , where the difference in cell density (N) between days  $t_2$  and  $t_1$  represents the greatest (positive) change in cell density throughout the duration of the experiment (Guillard, 1973). At specific time points, cell density (cells  $\text{mL}^{-1}$ ) was normalized to both ATX-A concentrations ( $\mu\text{g L}^{-1}$ ) and the amount of  $\text{N}_2$  fixed ( $\mu\text{mol N}_2\text{-fixed L}^{-1} \text{ day}^{-1}$ ) to determine cellular quotas of ATX-A and  $\text{N}_2$  fixation rates per cell.

Dissolved fixed N samples were collected by passing sample through a pre-combusted (450°C) glass fiber filter (GF/F) and stored frozen (-20°C) until analysis on a Lachat Instruments autosampler (ASX-520). Specifically, nitrate was analyzed using the Greiss reaction following reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  using cadmium columns (Jones, 1984), while ammonium was analyzed using a revised version of the indophenol blue method (Parsons et al., 1984).  $\text{NO}_3^-$  and  $\text{NH}_4^+$  analyses proceeded when the recovery of standard reference material

(SPEX CertiPrep™) was at least  $90 \pm 10\%$ . To measure  $F_v/F_m$ , or the maximum quantum efficiency of photosystem (PS) II, dark-adapted *in vivo* ( $F_0$ ) and DCMU (3,4-dichlorophenyl-1,1-dimethylurea)-enhanced *in vivo* fluorescence ( $F_m$ ) was measured daily on a Turner Designs TD-700 fluorometer (Ex/Em = 340 – 500/>665 nm). Readings were blank-corrected using standard BG11 media (Harke and Gobler, 2013). DCMU blocks electron transfer between photosystems, stopping photosynthesis, and is a reliable indicator of stress in eukaryotic phytoplankton and cyanobacteria due to fixed N and/or P depletion (Parkhill et al., 2001; Simis et al., 2012). Samples were also collected from –N + P and –N–P cultures to measure alkaline phosphatase activity (APA), which is a reliable indicator of stress induced by the lack of  $\text{PO}_4^{3-}$  in aquatic systems (Dyhrman et al., 2007), every other day. APA was measured on a Turner Designs TD-700 fluorometer (Ex/Em = 300 – 400/410 – 600 nm) using 4-methylumbelliferone phosphate (250  $\mu\text{M}$ ) as the substrate (Hoppe, 1983). APA measured using this method correlates significantly with the expression of the gene *phoX* that encodes for alkaline phosphatase in cyanobacteria (Harke et al., 2012).

$\text{N}_2$  fixation rates were measured every other day using the acetylene reduction method (Capone, 1993). Briefly, acetylene ( $\text{C}_2\text{H}_2$ ) is reduced by nitrogenase to ethylene, as one molecule of  $\text{N}_2$  is fixed for every four  $\text{C}_2\text{H}_4$  molecules produced in *Dolichospermum* (Hardy et al., 1973; Jensen and Cox, 1983). Acetylene was made by reacting 7 grams of lab-grade calcium carbide (Fisher Scientific) with 700 mL of deionized water (Hyman and Arp, 1987; Beversdorf et al., 2013), with the resulting gas collected in Supelco Tedlar bags. The amount of ethylene produced was quantified using  $\text{C}_2\text{H}_4$  standards made by injecting 1% ethylene in  $\text{N}_2$  (Airgas) into air-tight vials immediately prior to sample analysis. Acetylene was injected into the headspace of air-tight 10 mL vials containing samples, which were then placed into the incubator with experimental cultures for 3–4 h, after which a portion of the headspace was extracted and injected into a Trace 1310 Gas Chromatograph (Thermo Scientific). The amount of ethylene produced was determined using Chromeleon Chromatography Data System (CDS) software (Version 7.3).

For the final timepoint (day 9) of the experiment, 25 mL of sample from each flask was collected for anatoxin analysis by filtering water through a pre-combusted GF/F and stored frozen at  $-20^\circ\text{C}$ . ATX-A was extracted using an acetonitrile: $\text{H}_2\text{O}$ :formic acid (80:19.9:0.1) mixture (Dell'Aversano et al., 2005). Extracts were then quantified via liquid chromatography tandem mass spectrometry (LC-MS/MS) using an Agilent 1200 series HPLC and Agilent 6410 triple quadrupole mass spectrometer equipped with a Peak Nitrogen generator #NM30LA (Peak Scientific, Inc. Billerica, MA). ATX-A was detected using qualifier and quantifier ions (Buckland et al., 2005; James et al., 2005) using Agilent

MassHunter Qualitative Analysis software (version B.03.01) and quantified by normalization to the parent compound.

## RNA isolation and sequencing

To characterize differential expression of the *Dolichospermum* sp. 54 transcriptome relative to the control, RNA was acquired using Millipore Sterivex filters (0.22  $\mu\text{m}$ ) at the end of the experiment (day 9), filtering 50 mL from each replicate per treatment ( $n = 3$ ). After filtration, samples were flash frozen in liquid  $\text{N}_2$  and stored at  $-80^\circ\text{C}$ . Total nucleic acids were extracted using the cetyltrimethyl ammonium bromide (CTAB) method (Dempster et al., 1999; Harke and Gobler, 2013). Briefly, 1 mL of CTAB lysis buffer was added to each frozen sample while still in the Sterivex filter, heated to  $50^\circ\text{C}$ , and returned to  $-80^\circ\text{C}$  overnight. After placing frozen samples in a water bath set at  $65^\circ\text{C}$ , 750  $\mu\text{L}$  of CTAB was carefully removed from each Sterivex filter using a syringe and transferred to an Eppendorf tube, centrifuged, extracted with 750  $\mu\text{L}$  chloroform, precipitated with an isopropanol/sodium chloride solution, and centrifuged one final time.

The quality and quantity of sample nucleic acids were assessed using a Nanodrop spectrophotometer and a Qubit fluorometer in tandem with a dsDNA BR Assay kit (ThermoFisher Scientific) following the manufacturer's instructions, then stored at  $-80^\circ\text{C}$ . Total nucleic acid samples were treated with RNase-free DNase (Qiagen) to digest genomic DNA, and resulting total RNA was quantified using a Qubit<sup>®</sup> fluorometer and quality assessed using an Agilent QC Bioanalyzer. A relatively low amount of RNA was extracted from one of the –N–P replicates ( $\sim 3 \text{ ng } \mu\text{L}^{-1}$ ) and was thus removed from analysis. Samples were then sent to Columbia University's Next Generation Sequencing facility for sequencing on an Illumina HiSeq 4000 system, prior to which ribosomal RNA was depleted and sequencing libraries were prepared using a TruSeq Ribo-Zero Gold kit.  $20 \times 10^6$  raw 100 bp single-end reads were sequenced per sample.

## Read mapping and analysis

The quality of the raw reads was assessed using FASTQC (version 0.11.5). Through Trimmomatic (version 0.39), quality trimming was conducted on raw reads to remove adapter sequences and poor quality bases (Bolger et al., 2014). Trimming parameters were set as follows: SLIDINGWINDOW:4:15 – ILLUMINACLIP:adaptor\_truseq.fasta:2:30:15 – MINLEN:30, with a custom made truseq adapter file. Cleaned, filtered, and trimmed reads were then assembled using Trinity (version v2.8.4) (Grabherr et al., 2011; Haas et al., 2013); the transcriptome assembly was evaluated using RNA-Seq. Clean reads from different treatments were first pooled and

normalized using Trinity's *in silico* normalization module, then assembled *de novo* into transcripts using Trinity's single-end mode with default settings. The candidate open reading frames (ORFs) and deduced amino acid sequences were obtained using TransDecoder (version 5.5.0<sup>1</sup>) using search results of the BLASTP (BLAST + version 2.9.0) and Hmmscan (HMMER version 3.2.1) databases. Diamond BLASTX was used to annotate transcripts against the UniProt\_Swiss-Prot database (release 2020.01), using the genome of *Anabaena* sp. WA102 as a reference, and an e-value of  $1e^{-3}$  to avoid getting low annotations and mismatches.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to obtain KEGG orthology (KO) gene annotations from the UniProt\_Swiss-Prot database. KEGG annotations were also obtained through the GHOSTX (Suzuki et al., 2014) search of KOALA (KEGG Orthology And Links Annotation) (Kanehisa et al., 2014). Both sets of KEGG IDs were then merged to get the maximum number of gene annotations. Read counts of transcripts were obtained with Kallisto (version 0.46.0) using default settings (Bray et al., 2016) by mapping the clean reads of each sample to the assembled transcriptome, and the transcript abundances were normalized to transcripts per million (TPM) values. In Sleuth (version 0.30.0), Kallisto counts were used to characterize differential gene expression, then aggregate normalized counts by KO ID (Pimentel et al., 2017). Gene transcripts were quantified using units of scaled reads per base, and Wald tests were performed in Sleuth to determine whether the experimental groups exhibited significant (false discovery rate/*qval* < 0.05) differential gene expression relative to the control (-N + P), which was determined based on beta (b) - values generated in Sleuth that represent biased estimators of  $\log_2$ -fold changes ( $\Delta$ ) in transcript abundance. A gene ontology (GO) enrichment analysis was also performed on the Kallisto counts to map transcripts to genes associated with certain biological, cellular, and molecular categories of genes associated with N and P metabolism, growth, and photosynthesis. Differential expression at the transcript level for each treatment relative to the control was first determined using Sleuth, then processed and mapped to gene categories using the GOStats (version 2.56.0) package (Falcon and Gentleman, 2007).

To characterize differential expression of genes associated with anatoxin synthesis, Trimmomatic reads were assembled into transcripts using SPAdes (version 3.11.1) (Bankevich et al., 2013; Nurk et al., 2013; Bushmanova et al., 2019) and annotated using Prokka (version 1.12<sup>2</sup>) (Seeman, 2014). The annotated assembly and the *Anabaena* sp. 37 ATX-A - encoding gene sequences available on NCBI (Rantala-Ylinen et al., 2011), were then put through BLASTP to find which sequences in

the assembly matched the genes belonging to the anatoxin synthetase gene (*ana*) cluster.

## Statistical analyses and data visualization

Statistical analyses were done in R (Version 4.0.3) and graphing was done in both R and Microsoft Excel (Version 16.51).  $F_v/F_m$  and  $N_2$  fixation rate values for each replicate at each time point were averaged prior to being averaged with the values of other replicates from the same treatment. Using the dplyr package, a two-way analysis of variance (ANOVA) was performed to determine whether fixed N and P availability significantly affected growth rate,  $F_v/F_m$ , dissolved nutrient levels, ATX-A cell quota,  $N_2$  fixation rate, and normalized counts of *ana* transcripts. Tukey's HSD tests using the agricolae package were then performed *post hoc* to determine whether differences among treatments were significantly different to each other. For two-way ANOVAs, the Shapiro-Wilk and Fligner-Killeen tests were used to confirm that the data passed normality and homogeneity of variance, respectively. Transcript data for *anaE* and *anaF* exhibited non-normal distribution under Shapiro-Wilk but did exhibit normality ( $p > 0.05$ ) using Kolmogorov-Smirnov (KS) non-parametric tests. Significant differences in APA activity were compared between the control (-N + P) and -N-P across time using a Student's paired *t*-test. Linear regression analyses (F-tests) were performed using the stats package to determine whether there were correlations between  $N_2$  fixation and growth rate, and to determine whether ATX-A content covaried with nitrogenase activity and growth rate. In all cases, an alpha level of 0.05 was utilized.

To visualize the overall expression of genomes among treatments, a non-metric multidimensional scaling (nMDS) plot of TPM values was created using the vegan package (Version 2.5-7<sup>3</sup>). To test for statistically significant differences between treatments, 95% confidence intervals (CIs) were drawn around experimental groups in the nMDS plot. Furthermore, a two-way permutational multivariate analysis of variance (PERMANOVA) was performed on a Bray-Curtis dissimilarity matrix using the adonis2 package to determine whether N and/or P availability significantly influenced global gene expression. As stated before (see *Read mapping and analysis*), Wald tests were also performed using the sleuth package to determine whether the experimental groups exhibited significant (false discovery rate/*qval* < 0.05) differential gene expression relative to the control (-N + P). Global results in differential expression relative to the control were visualized using Bland-Altman (MA) plots, with b-values, or  $\log_2$ -fold changes ( $\Delta$ ) in expression, plotted as

<sup>1</sup> <https://transdecoder.github.io/>

<sup>2</sup> <https://vicbioinformatics.com/software/prokka.shtml>

<sup>3</sup> <https://github.com/vegandevs/vegan>

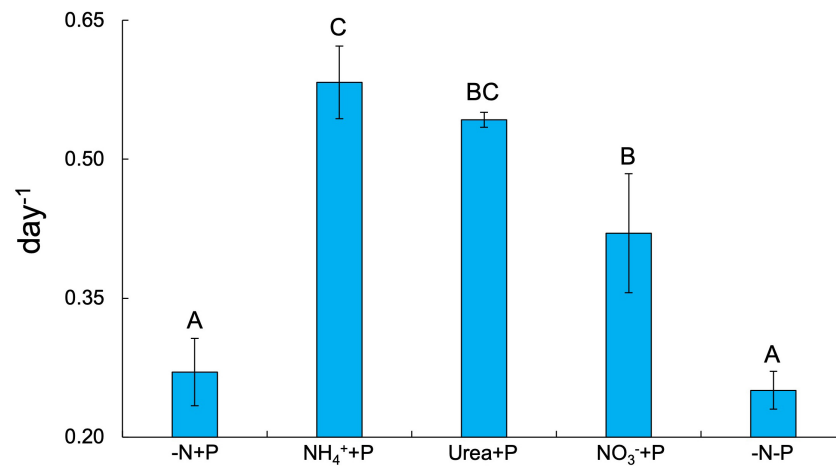


FIGURE 1

Maximum growth rates ( $\mu_{max}$ ; day<sup>-1</sup>) across all experimental groups. Error bars represent standard deviation. Letters above bars represent significant differences between experimental groups (Two-way ANOVA; Tukey's HSD *post hoc*;  $p < 0.05$ ).

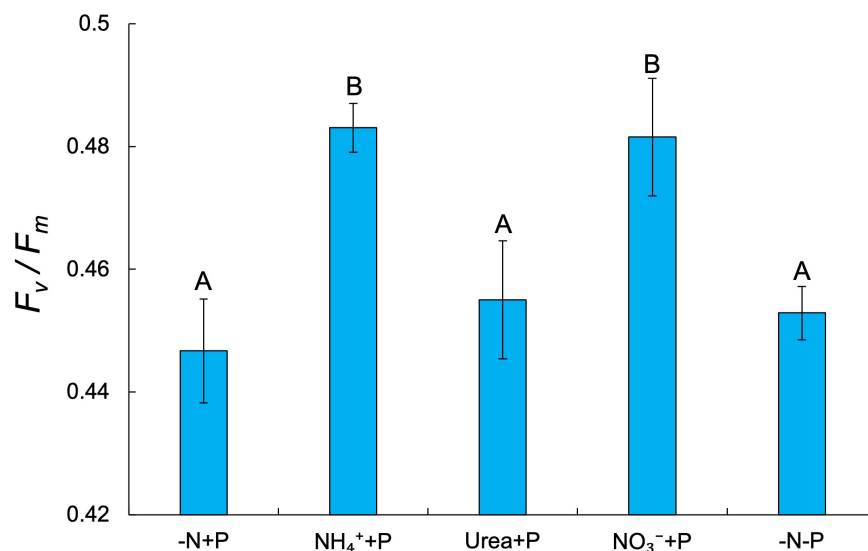


FIGURE 2

Photosystem II photosynthetic efficiency ( $F_v/F_m$ ) across treatments. Error bars represent standard deviation. Letters above bars represent significant differences between experimental groups (Two-way ANOVA; Tukey's HSD *post hoc*;  $p < 0.05$ ).

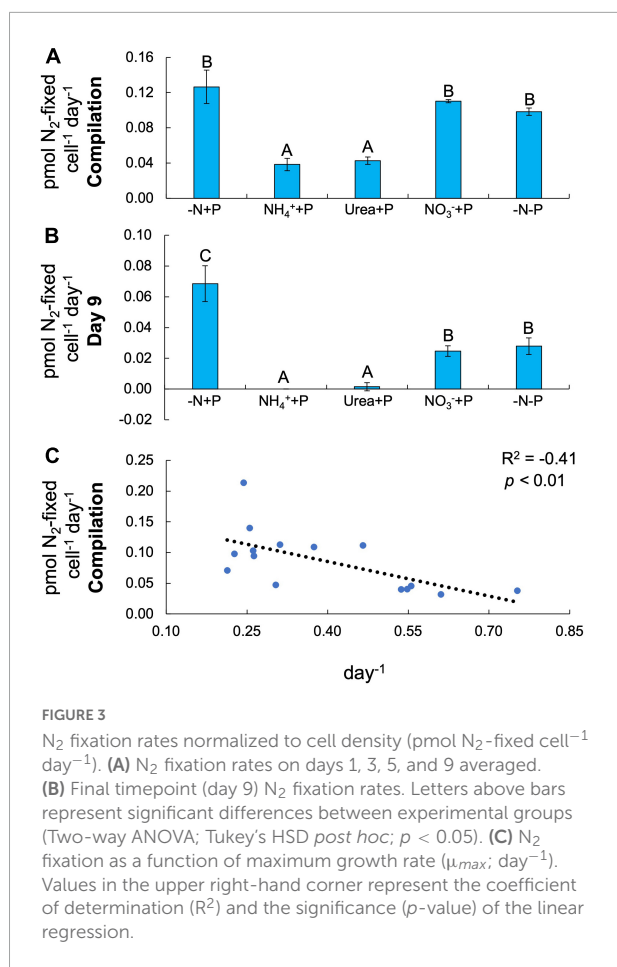
a function of mean abundance of annotated gene transcripts. The data were further examined for genes associated with nitrogen metabolism/fixation and phosphorus assimilation using the software Morpheus (Broad Institute<sup>4</sup>) to generate heatmaps. Hypergeometric distribution tests ( $p_{adj} < 0.05$ ) were performed using the GOstats package to determine whether gene categories associated with growth, N and P assimilation, and photosynthesis were significantly enriched relative to the control.

<sup>4</sup> <https://software.broadinstitute.org/morpheus/>

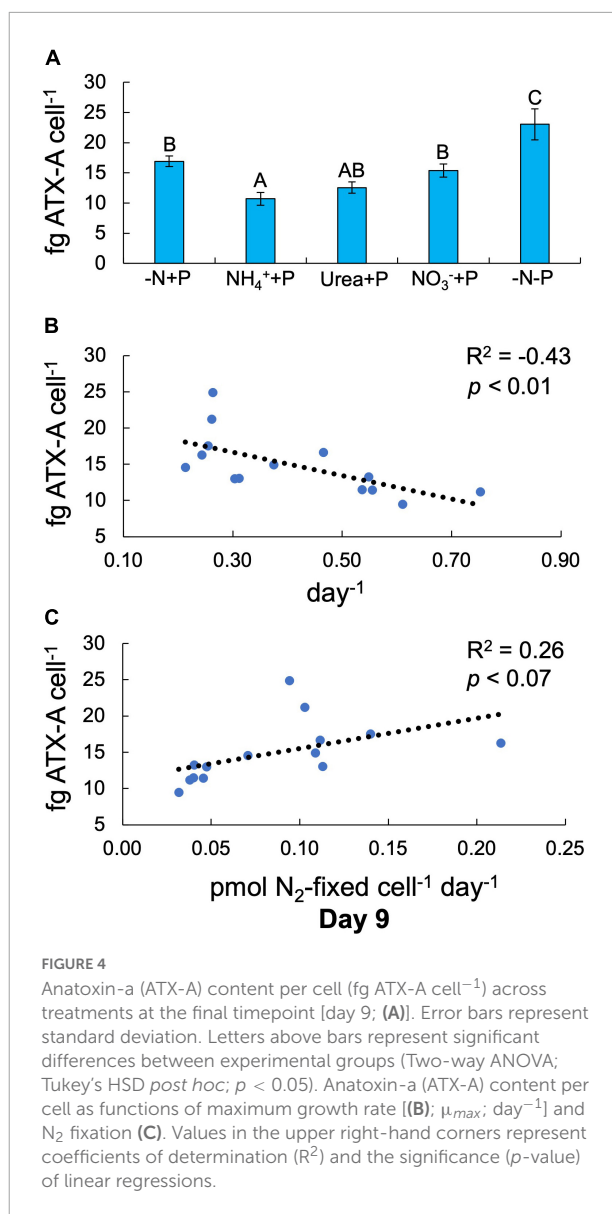
## Results

### Growth rate, nutrient availability, and physiological indicators of nutrient acquisition and stress

Fixed N availability alone had a significant positive effect on the maximum growth rates ( $\mu_{max}$  = day<sup>-1</sup>) of *Dolichospermum* sp. 54 (Two-way ANOVA;  $p < 0.001$ ), as cultures amended with NH<sub>4</sub><sup>+</sup> ( $0.58 \pm 0.04$  day<sup>-1</sup>), urea ( $0.54 \pm 0.01$  day<sup>-1</sup>), and NO<sub>3</sub><sup>-</sup> ( $0.42 \pm 0.06$



$\text{day}^{-1}$ ) all exhibited significantly higher  $\mu_{max}$  than those of the control ( $-N + P$ ;  $0.27 \pm 0.04 \text{ day}^{-1}$ ) and  $-N-P$  ( $0.25 \pm 0.02 \text{ day}^{-1}$ ) cultures (Tukey's HSD;  $p < 0.05$ ; **Figure 1**).  $\text{NH}_4^+ + P$  cultures exhibited significantly faster growth rates relative to  $\text{NO}_3^- + P$  cultures (Tukey's HSD;  $p < 0.05$ ; **Figure 1**). Dissolved  $\text{NO}_3^-$  and  $\text{NH}_4^+$  concentrations on days 6 and 7 of the experiment affirmed that the growth rates of fixed N-amended cultures were not limited by fixed N supply (**Supplementary Table 1**). PS-II photosynthetic efficiency ( $F_v/F_m$ ) was significantly altered by the availability of fixed N (Two-way ANOVA;  $p < 0.001$ ), but not P, though only two of the fixed N-enriched treatments,  $\text{NH}_4^+ + P$  ( $0.48 \pm 0.004 \text{ day}^{-1}$ ) and  $\text{NO}_3^- + P$  ( $0.48 \pm 0.01 \text{ day}^{-1}$ ), exhibited significantly (Tukey's HSD;  $p < 0.05$ ) higher average  $F_v/F_m$  values than  $-N + P$  cultures ( $0.45 \pm 0.01$ ; **Figure 2**). Furthermore, on days 3-8 of the experiment,  $F_v/F_m$  values of  $\text{NH}_4^+ + P$  and  $\text{NO}_3^- + P$  were higher than those of urea + P cultures (**Supplementary Figure 2**).  $-N-P$  cultures exhibited significantly (Student's paired *t*-test;  $p < 0.05$ ) greater APA activities relative to the



control during all time points measured (**Supplementary Figure 3**).

## $N_2$ fixation rates

Both fixed N (Two-way ANOVA;  $p < 0.001$ ) and P ( $p < 0.01$ ) availability significantly altered  $N_2$  fixation rates in *Dolichospermum* sp. 54 (**Figure 3A**). Amendments with reduced N compounds significantly decreased (Tukey's HSD;  $p < 0.05$ ) the average  $N_2$  fixation rate ( $\text{NH}_4^+ + P = 0.04 \pm 0.01 \text{ pmol } N_2\text{-fixed cell}^{-1} \text{ day}^{-1}$ ; urea + P =  $0.04 \pm 0.004 \text{ pmol } N_2\text{-fixed cell}^{-1} \text{ day}^{-1}$ ) relative to that of control cultures ( $-N + P$ ;  $0.13 \pm 0.02 \text{ pmol } N_2\text{-fixed cell}^{-1} \text{ day}^{-1}$ ; **Figure 3A**). On the

final day of the experiment,  $\text{NO}_3^- + \text{P}$  ( $0.02 \pm 0.003$  pmol  $\text{N}_2$ -fixed  $\text{cell}^{-1} \text{day}^{-1}$ ) and  $-\text{N}-\text{P}$  ( $0.03 \pm 0.01$  pmol  $\text{N}_2$ -fixed  $\text{cell}^{-1} \text{day}^{-1}$ ) cultures exhibited significantly (Tukey's HSD;  $p < 0.05$ ) lower  $\text{N}_2$  fixation rates relative to the control ( $0.07 \pm 0.01$  pmol  $\text{N}_2$ -fixed  $\text{cell}^{-1} \text{day}^{-1}$ ) and significantly higher  $\text{N}_2$  fixation rates relative to  $\text{NH}_4^+ + \text{P}$  (below detection limit) and urea + P ( $0.002 \pm 0.003$  pmol  $\text{N}_2$ -fixed  $\text{cell}^{-1} \text{day}^{-1}$ ) cultures (Figure 3B). Across all cultures, there was a significant negative correlation between  $\text{N}_2$  fixation and growth rates (F-test;  $R^2 = -0.41$ ,  $p < 0.01$ ; Figure 3C).

## Anatoxin-a cell quotas

Fixed N (Two-way ANOVA;  $p < 0.001$ ) and P ( $p < 0.01$ ) availability both significantly altered cellular ATX-A content (Figure 4). Of all experimental treatments, only  $\text{NH}_4^+ + \text{P}$  and  $-\text{N}-\text{P}$  exhibited significantly lower ( $10.7 \pm 1.06$  fg ATX-A  $\text{cell}^{-1}$ ) and higher ( $23.0 \pm 2.59$  fg ATX-A  $\text{cell}^{-1}$ ) cellular ATX-A quotas than the control ( $-\text{N} + \text{P}$ ;  $16.9 \pm 0.88$  fg ATX-A  $\text{cell}^{-1}$ ), respectively (Tukey's HSD;  $p < 0.05$ ; Figure 4A). ATX-A content was significantly (F-test;  $R^2 = -0.43$ ,  $p < 0.01$ ; Figure 4B) negatively correlated with growth rate, while ATX-A content exhibited a positive, marginally significant (F-test;  $R^2 = 0.26$ ,  $p < 0.07$ ; Figure 4C) correlation with  $\text{N}_2$  fixation rate.

## Global transcriptomic changes

Non-metric multi-dimensional (nMDS) scaling of normalized read counts mapped to the Trinity assembly revealed considerable overlap among transcriptomes, particularly with respect to  $\text{NH}_4^+ + \text{P}$ , which overlapped with urea + P and the control group ( $-\text{N} + \text{P}$ ; Figure 5). The transcriptomes of  $-\text{N}-\text{P}$  cultures, in contrast, were significantly different from those of all other treatments (CI = 95%;  $p < 0.05$ ) except those of  $\text{NH}_4^+ + \text{P}$ . Furthermore, the transcriptomes of  $\text{NO}_3^- + \text{P}$  were significantly different from those of  $\text{NH}_4^+ + \text{P}$  and  $-\text{N}-\text{P}$  cultures (CI = 95%;  $p < 0.05$ ). Fixed N ( $p = 0.001$ ) and P ( $p = 0.05$ ) availability significantly (Two-way PERMANOVA) influenced differences in global gene expression among treatments. All treatments displayed substantially more annotated transcripts that were significantly lower in abundance than transcripts that were significantly higher in abundance relative to the control (Wald test;  $qval < 0.05$ ; Figure 6). The transcriptomes of  $\text{NH}_4^+ + \text{P}$ ,  $\text{NO}_3^- + \text{P}$ , and urea + P had fewer than 100 transcripts that were significantly more abundant and  $\sim 630 - 810$  transcripts that were significantly less abundant (Figures 6A–C). The transcriptomes of  $-\text{N}-\text{P}$  had the largest number of differentially expressed transcripts (Wald test;  $qval < 0.05$ ) with  $\sim 400$  and  $\sim 1200$  transcripts significantly more and less abundant, respectively (Figure 6D).

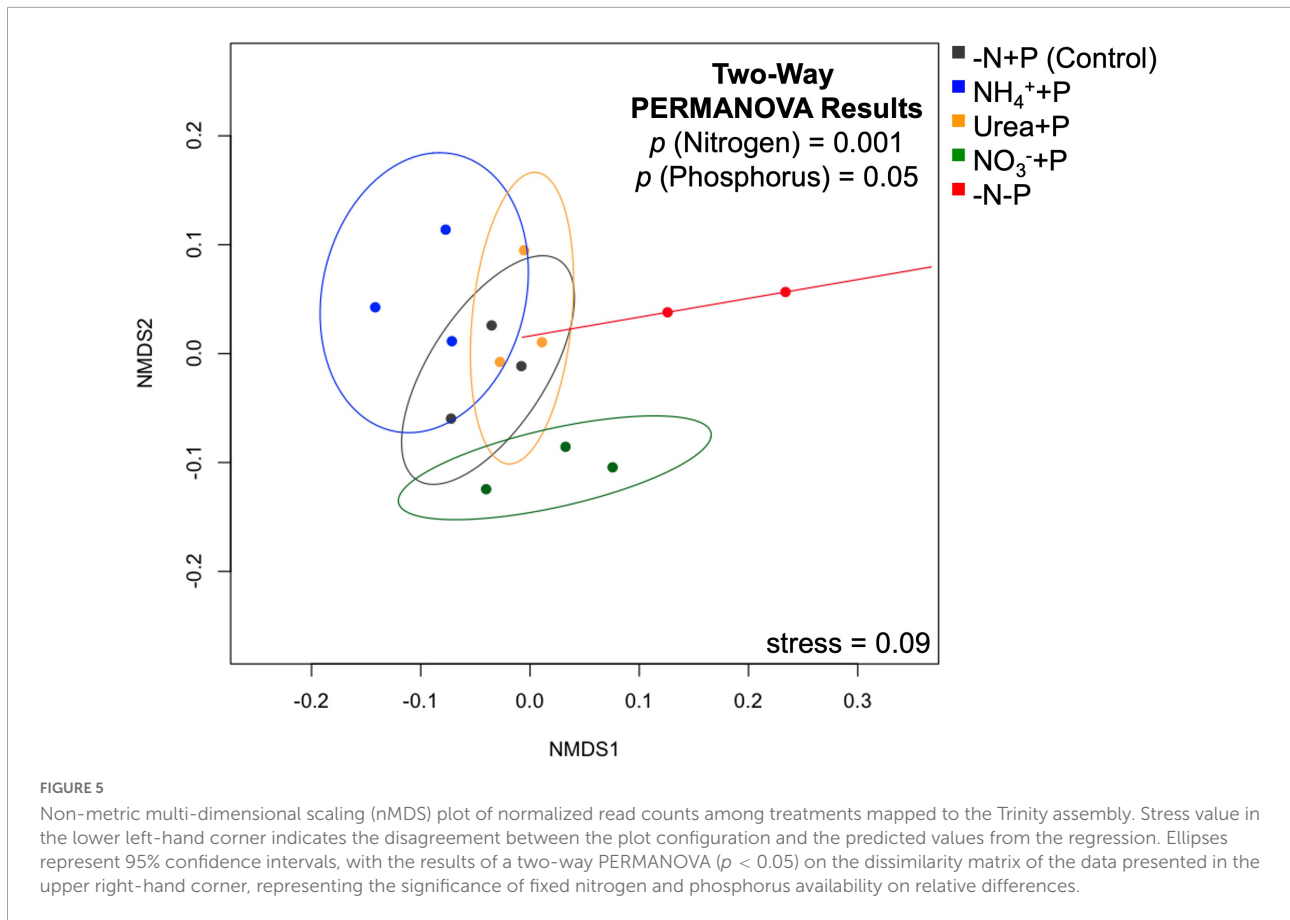
## Enriched gene categories and differentially expressed genes

Among experimental treatments, the number of significantly downregulated gene categories (hypergeometric distribution analysis;  $padj < 0.05$ ) relative to the control ( $-\text{N} + \text{P}$ ) was higher than the number of upregulated gene categories (Figure 7). The transcriptomes of  $\text{NH}_4^+ + \text{P}$  exhibited the smallest number of significantly downregulated gene categories associated with fixed N and P transport/metabolism (11; Figure 7A). Transcriptomes in this treatment also had the second highest number of significantly upregulated gene categories (9) after those of  $\text{NO}_3^- + \text{P}$  (11; Figure 7B). Only the transcriptomes of  $\text{NH}_4^+ + \text{P}$  and urea + P cultures exhibited significant downregulation of organonitrogen biosynthesis, cellular N metabolism, and amide biosynthesis gene categories; these gene categories exhibited an average number of  $\sim 300$ ,  $\sim 500$ ,  $\sim 200$  differentially expressed genes (DEGs), respectively, in both treatments. These genes comprised roughly 18% of the total number (5597) of DEGs that were downregulated across all experimental treatments (Figure 7A). Of these,  $\text{NH}_4^+ + \text{P}$  exhibited significant upregulation of 5 out of 7 gene categories associated with N metabolism and biosynthesis, while only 2 and 1 of these gene categories were upregulated in urea + P and  $\text{NO}_3^- + \text{P}$ , respectively, and  $-\text{N}-\text{P}$  exhibited no such enrichment (Figure 7B).  $\text{NO}_3^- + \text{P}$  and  $-\text{N}-\text{P}$  were the only treatments to exhibit significant downregulation of cellular N biosynthesis and cellular amide metabolism gene categories; both gene categories in  $\text{NO}_3^- + \text{P}$  and  $-\text{N}-\text{P}$  exhibited  $\sim 200$  DEGs (Figure 7A).

Across all experimental treatments, a majority ( $> 90\%$ ) of annotated transcripts associated with N metabolism were significantly lower (Wald test;  $qval < 0.05$ ) in abundance relative to the control ( $-\text{N} + \text{P}$ ; Figure 8). Genes involved in  $\text{NO}_3^-$  metabolism (*narB*, *narG*, *narL*) were significantly differentially expressed in at least one of the experimental treatments.  $\text{NO}_3^- + \text{P}$  ( $\Delta \cong +1.11$ ,  $qval < 0.01$ ) and  $-\text{N}-\text{P}$  ( $\Delta \cong +1.38$ ,  $qval < 0.01$ ) cultures significantly increased the number of transcripts for *narB*, which encodes for ferredoxin–Nitrate reductase. The number of transcripts for *narL*, which encodes for the  $\text{NO}_3^-/\text{NO}_2^-$  response regulator, and *narG*, which encodes the  $\Delta$ -subunit for  $\text{NO}_3^-/\text{NO}_2^-$  oxidoreductase, were, respectively, significantly lower in  $\text{NH}_4^+ + \text{P}$  ( $\Delta \cong -1.89$ ,  $qval < 0.05$ ) and urea + P ( $\Delta \cong -0.70$ ,  $qval < 0.05$ ) transcriptomes (Figure 8).

The number of transcripts for *ureA*, which encodes for a subunit of urease, and *urtC*, which encodes for the urea transport system permease, significantly (Wald test;  $qval < 0.05$ ) increased in the transcriptomes of  $\text{NO}_3^- + \text{P}$  ( $\Delta \cong +1.60$ ) and  $-\text{N}-\text{P}$  ( $\Delta \cong +0.58$ ), respectively, relative to the control ( $-\text{N} + \text{P}$ ; Figure 8). In contrast, *ureA* exhibited a significantly lower number of transcripts in the transcriptomes of  $\text{NH}_4^+ + \text{P}$  ( $\Delta \cong -2.68$ ,  $qval < 0.001$ ), as did *ureC* ( $\Delta \cong -1.23$ ,  $qval < 0.05$ ),

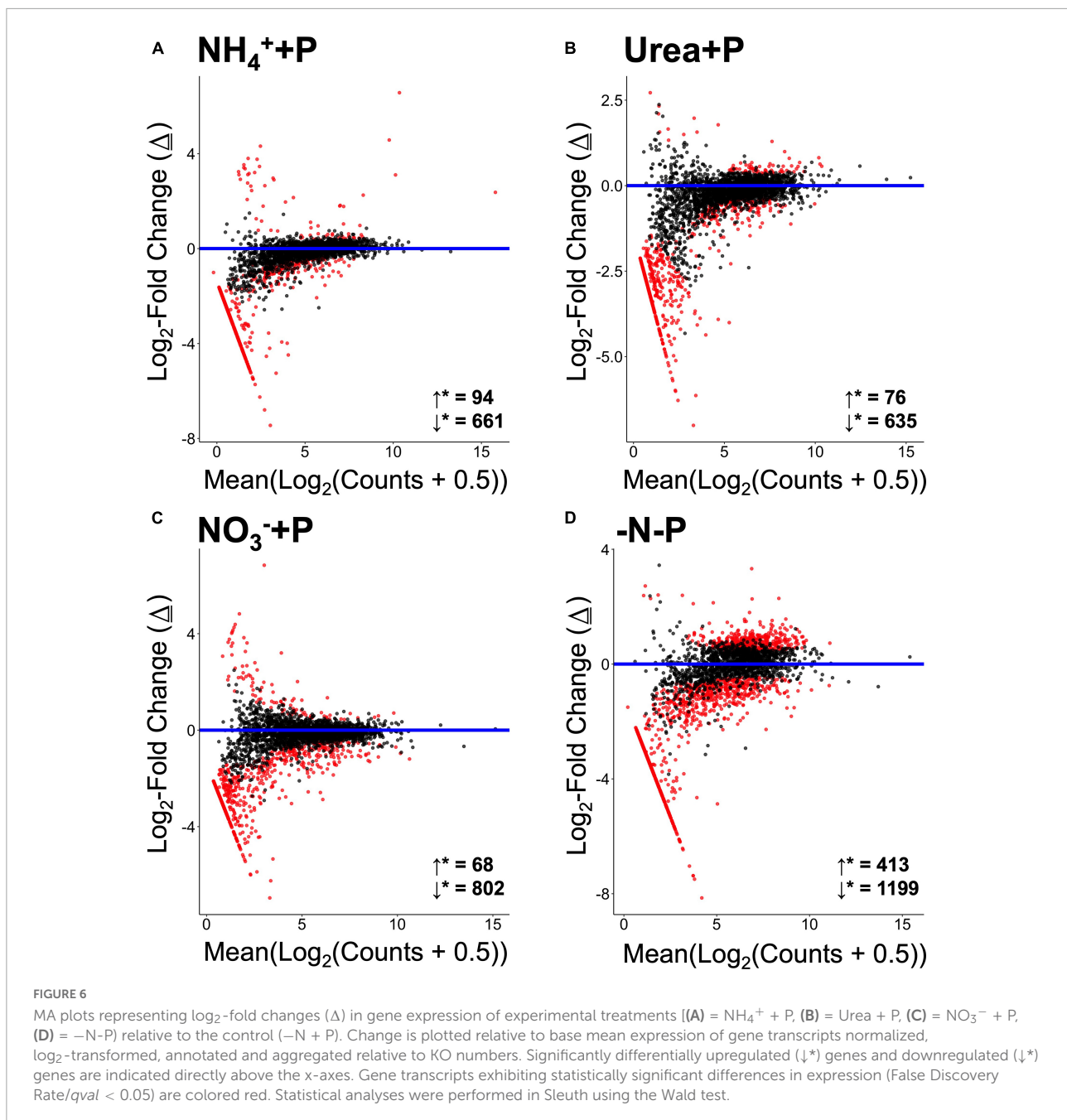




and other genes that encode for enzymes associated with urea degradation, namely *atzF* (allophanate hydrolase;  $\Delta \cong -1.20$ ,  $qval < 0.05$ ) and *E6.3.4.6* (urea carboxylase;  $\Delta \cong -3.62$ ,  $qval < 0.05$ ). Significant differential expression ( $\Delta \cong -1.58$ ,  $qval < 0.05$ ) of *amt1*, which encodes for an ammonium transporter, was only observed in the transcriptomes of  $\text{NH}_4^+$  + P, with transcripts being lower in abundance. Other N metabolism genes that were differentially expressed include *cphA*, which encodes for cyanophycin synthetase, exhibited a significantly lower number of transcripts in the transcriptomes of  $\text{NH}_4^+$  + P ( $\Delta \cong -0.67$ ,  $qval < 0.05$ ), as well as genes that encode for cyanase, *cynB* ( $\Delta \cong -1.31$ ,  $qval < 0.05$ ) and *cynR* ( $\Delta \cong -1.88$ ,  $qval < 0.05$ ), which exhibited significantly lower transcripts in the transcriptomes of -N-P. The number of transcripts for glutamine synthetase-encoding genes (*gln*) was significantly lower in the transcriptomes of  $\text{NH}_4^+$  + P (*glnB*;  $\Delta \cong -0.70$ ,  $qval < 0.05$ ),  $\text{NO}_3^-$  + P (*glnL*;  $\Delta \cong -1.12$ ,  $qval < 0.01$ ), and -N-P (*glnL*;  $\Delta \cong -1.13$ ,  $qval < 0.05$ ), while *gltD* was the only gene involved in glutamate synthesis to exhibit a significantly lower transcript abundance, specifically in the transcriptomes of urea + P ( $\Delta \cong -1.35$ ,  $qval < 0.001$ ). Significantly lower numbers of transcripts (Wald test;  $qval < 0.001$ ) of *nifE*, *nifV*, and *nifW*, which encode for nitrogenase subunits, were observed

in all experimental treatments (Figure 8). Significantly lower numbers of transcripts of *nifB* ( $\Delta \cong -1.52$ ,  $qval < 0.001$ ) and *nifD* ( $\Delta \cong -1.88$ ,  $qval < 0.001$ ) were observed in  $\text{NH}_4^+$  + P, while the former and latter genes were present at significantly lower transcript abundances in  $\text{NO}_3^-$  + P ( $\Delta \cong -0.64$ ,  $qval < 0.05$ ) and urea + P ( $\Delta \cong -1.62$ ,  $qval < 0.05$ ), respectively, compared to the control. Finally, the transcriptomes of -N-P exhibited significantly lower transcript abundance of *fixL* ( $\Delta \cong -2.15$ ,  $qval < 0.001$ ; Figure 8), which encodes for a putative oxygen sensor.

Genes associated with phosphorus (P) assimilation that were differentially expressed relative to the control (-N + P) included those that belong to the *phn*, *pho*, and *pst* clusters (Figure 9). For cultures amended with fixed N, genes that exhibited a significant change (Wald test;  $qval < 0.05$ ) in transcript abundance were always lower than those of the control, while the transcriptomes of -N-P were the only ones to exhibit a significantly greater number of transcripts relative to the control, specifically for four *phn* genes and *pstC* (Figure 9). While non-significant, polyphosphate synthesizing (*ppk1*,  $\Delta \cong +0.47$ ) and degradation (*ppx*,  $\Delta \cong +0.49$ ) - encoding genes also exhibited the highest average increase in transcript number in the transcriptomes of -N-P than any other treatment relative to the control (Wald test;  $q > 0.05$ ; Figure 9).



Of the genes associated with ATX-A synthesis, transcript abundances of *anaA*, *anaE*, and *anaF* were significantly (Two-Way ANOVA;  $p < 0.05$ ) altered by P availability, while *anaC* transcript numbers were significantly affected by fixed N and P availability (Figure 10). -N-P cultures exhibited a significantly (Tukey HSD;  $p < 0.05$ ) higher number of *anaA* transcripts ( $7.0 \pm 0.0$ ) relative to those of the control (-N + P;  $5.3 \pm 0.6$ ) as well as NH<sub>4</sub><sup>+</sup> + P ( $5.0 \pm 0.0$ ) and urea + P ( $5.3 \pm 0.6$ ) cultures, while the number of *anaE* transcripts in -N-P cultures ( $10.0 \pm 0.0$ ) was significantly higher than those of the control and NH<sub>4</sub><sup>+</sup> + P ( $8.7 \pm 0.6$ ). The number of *anaC*

transcripts was highest in -N-P cultures ( $8.0 \pm 0.0$ ) and was significantly higher than that of NH<sub>4</sub><sup>+</sup> + P cultures ( $5.5 \pm 0.7$ , Figure 10).

## Discussion

This study characterized the effects of NH<sub>4</sub><sup>+</sup>, urea, and NO<sub>3</sub><sup>-</sup> as well as P availability on *Dolichospermum* sp. 54. Relative to the control (-N + P), reduced N species significantly increased growth rates and decreased N<sub>2</sub> fixation rates

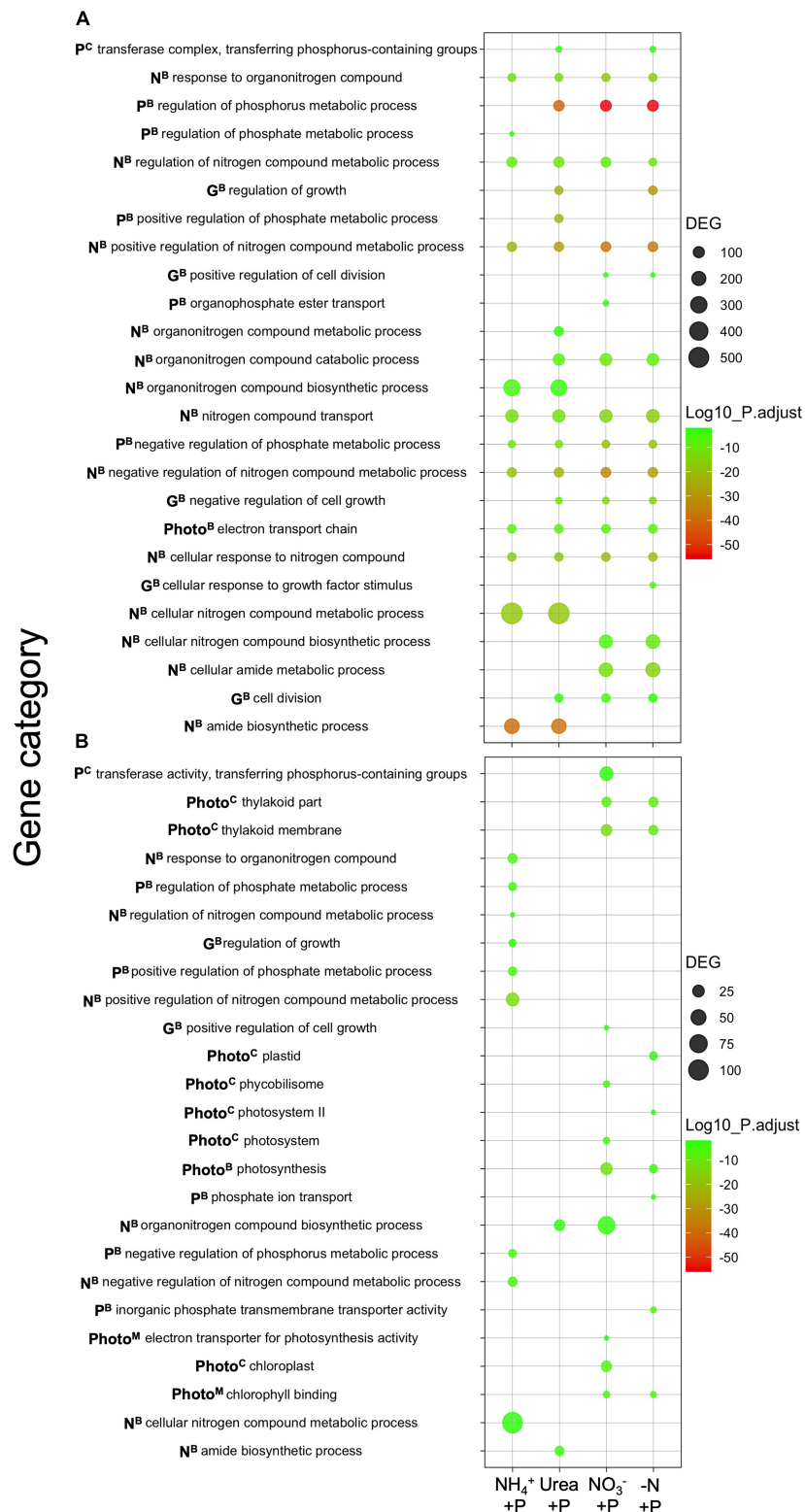


FIGURE 7

Differentially expressed categories of genes [(A) = 4-fold downregulated, (B) = 4-fold upregulated] relative to the control (-N + P). Results are from transcript-based Gene Ontology (GO) enrichment analysis. The significant differential expression of genes was determined using Sleuth (Wald test; *qval* < 0.05), and gene categories were tested for significant differential expression using GOenrich (hypergeometric distribution analysis; *padj* < 0.05). *Padj* values were Log<sub>10</sub>-transformed for scaling purposes, and green - red colorations corresponded to lower - higher degrees of significance. Gene categories plotted were those associated with nitrogen (N) and phosphorus (P) assimilation, growth (G), as well as photosynthesis (Photo), while superscripts correspond to biological (<sup>B</sup>), cellular (<sup>C</sup>), and molecular (<sup>M</sup>) gene categories. The number of differentially expressed genes (DEGs) in gene categories are represented by circle size.



(NH<sub>4</sub><sup>+</sup> + P and urea + P), ATX-A cellular quotas (NH<sub>4</sub><sup>+</sup> + P), as well as *nif* (NH<sub>4</sub><sup>+</sup> + P and urea + P) transcript abundances. P depletion did not affect growth rate but significantly enhanced ATX-A cellular content and *ana* transcript abundance. We also found a significant negative correlation between ATX-A concentration and growth rate.

## Effects of fixed N and P availability on growth and photosynthetic activity

*Dolichospermum* sp. 54 amended with fixed N exhibited significantly higher maximum growth rates (NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, urea) and  $F_v/F_m$  (NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>) relative to the control ( $-N + P$ ) and  $-N-P$  cultures. NH<sub>4</sub><sup>+</sup> + P and NO<sub>3</sub><sup>-</sup> + P were also the only experimental treatments to exhibit significant upregulation of gene categories associated with growth. It has been well established with other *Dolichospermum* (Rhee and Lederman, 1983; Mishra, 1997; Zulkefli and Hwang, 2020) and Nostocales (Presing et al., 1996; Ammar et al., 2014) isolates that NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> significantly increase growth and decrease N<sub>2</sub> fixation rates, though NH<sub>4</sub><sup>+</sup> is generally considered more effective than NO<sub>3</sub><sup>-</sup> in affecting these physiological processes (Stacey et al., 1977; Elder and Parker, 1984). This is not always the case, however, as some members of this order may prefer NO<sub>3</sub><sup>-</sup> and urea (Stucken et al., 2014; Qian et al., 2017; Erratt et al., 2018) or even fixed N-deplete conditions (Velzeboer et al., 2001; Molot, 2017) over NH<sub>4</sub><sup>+</sup> for growth. The considerable diversity in responses to fixed N among Nostocales stresses the importance of determining how multiple fixed N compounds affect other members of this order, particularly toxin producers such as *Dolichospermum* sp. 54. NO<sub>3</sub><sup>-</sup> must be reduced to nitrite (NO<sub>2</sub><sup>-</sup>) and again to NH<sub>4</sub><sup>+</sup> before entering anabolic pathways (Herrero and Flores, 2018). The  $F_v/F_m$  of NO<sub>3</sub><sup>-</sup> + P was not significantly different from that of NH<sub>4</sub><sup>+</sup> + P and was the only fixed N-amended treatment to exhibit significant upregulation of photosynthetic gene categories. It is plausible that a significant fraction of the reductants produced by the photosynthetic light reactions in this treatment were consumed by NO<sub>3</sub><sup>-</sup> reduction, thus keeping photosynthetic efficiency high, but resulting in a lowered growth rate relative to NH<sub>4</sub><sup>+</sup>-grown cultures (Flores et al., 1983, 2005).

Urea + P cultures exhibited growth rates that were significantly enhanced relative to the control but not significantly different than those of NH<sub>4</sub><sup>+</sup> + P or NO<sub>3</sub><sup>-</sup> + P. Strains of CHAB taxa such as *Microcystis* and *Dolichospermum* grown on urea often exhibit enhanced growth rates, toxin production, and/or amino acid biosynthetic rates relative to cells grown with NH<sub>4</sub><sup>+</sup> or NO<sub>3</sub><sup>-</sup> (Qian et al., 2017; Erratt et al., 2018; Krausfeldt et al., 2019). Aside from two NH<sub>3</sub> molecules, urea hydrolysis also yields one CO<sub>2</sub> molecule (Mobley and Hausinger, 1989; Valladares et al., 2002; Veaudor et al., 2019). While urea is potentially both a source of fixed N and carbon

for cyanobacterial growth (Krausfeldt et al., 2019), our findings suggest that the increase in *Dolichospermum* sp. 54 growth on this reduced N compound was largely due to the ability to utilize  $\text{NH}_3$  rather than  $\text{CO}_2$ .

## Fixed N and P availability effects on $\text{N}_2$ fixation

While  $\text{N}_2$  fixation can give *Dolichospermum* spp. and other Nostocales taxa a competitive edge over non-diazotrophic cyanobacteria under fixed N-limiting conditions, nitrogenase activity is an energetically demanding process that generally comes at the cost of higher growth rates (Willis et al., 2016; Herrero and Flores, 2018; Wannicke et al., 2021). Traditionally speaking, the productivity of non-diazotrophic phytoplankton was thought to be primarily limited by P (Smith, 1983, 2016; Schindler et al., 2008). Considerable evidence collected over the last decade or so indicates that this is not necessarily the case, as  $\text{N}_2$  fixation alone is insufficient to supply CHAB taxa with N levels sufficient in order to initiate blooms (Scott and McCarthy, 2010; Hellweger et al., 2016; Hayes et al., 2019). While this concept is primarily applied to non-diazotrophic cyanobacteria, our findings indicate that diazotrophs such as *Dolichospermum* exhibit a substantially greater preference for fixed N assimilation over nitrogenase activity, and thus likely play a markedly less important role in supplying newly fixed N to nitrogen-replete freshwater ecosystems during bloom events. The significantly enhanced growth rates of *Dolichospermum* sp. 54 cultured with  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , and urea reported in this study further complements the findings of other studies that have documented Nostocales blooms when fixed N concentrations are high (Chaffin et al., 2019, 2020; Gamez et al., 2019).

$\text{N}_2$  fixation rates of cultures amended with reduced fixed N ( $\text{NH}_4^+$  and urea) were significantly lower than those of any other group for the entire experiment, while the  $\text{N}_2$  fixation rates of  $\text{NO}_3^- + \text{P}$  and  $-\text{N}-\text{P}$  were significantly higher than those of the reduced N treatments. These observations, as well as the significant negative correlation between  $\text{N}_2$  fixation rate and growth rate, were consistent with prior studies (Mickelson et al., 1967; Stacey et al., 1977; Ge et al., 1990; Mekonnen et al., 2002). Despite being grown under P-deplete conditions,  $-\text{N}-\text{P}$  cultures exhibited  $\text{N}_2$  fixation rates that were not different from those of the control. The  $-\text{N}-\text{P}$  cultures may have maintained relatively high  $\text{N}_2$  fixation rates over the course of the experiment by hydrolyzing internal P stores (Gerber and Wickstrom, 1990; Burut-Archanai and Powtongsook, 2017), as their transcriptomes exhibited the greatest increase in transcript abundance of polyphosphate synthesis (*ppk1*) and degradation (*ppx*) - encoding genes (Akiyama et al., 1993; Sanz-Luque et al., 2020) of the experimental treatments relative to the control. This, coupled with significantly enhanced APA

activity in  $-\text{N}-\text{P}$  relative to control ( $-\text{N} + \text{P}$ ) cultures, likely facilitated sustained growth and  $\text{N}_2$  fixation rates that matched the control during the experiment, and further reinforces the widely accepted notion that Nostocales such as *Dolichospermum* have multiple strategies to handle the stresses of P-limitation (Isvanovics et al., 2000; Wu et al., 2012; Lin et al., 2018).

The  $\text{N}_2$  fixation rates of  $\text{NO}_3^- + \text{P}$  cultures were also similar to those of  $-\text{N}$  treatments when averaged over the entire experiment, strongly indicating that  $\text{NO}_3^-$  assimilation and nitrogenase activity are utilized simultaneously (Bone, 1971; Elder and Parker, 1984). Supporting this notion, other *Dolichospermum* strains were still reported to exhibit detectable nitrogenase activities when grown at particularly high (1–10 mM)  $\text{NO}_3^-$  concentrations, whereas those grown at the same or lower concentrations of  $\text{NH}_4^+$  did not. These studies did not, however, report significantly enhanced growth rates in *Dolichospermum* amended with  $\text{NH}_4^+$  relative to those grown on  $\text{NO}_3^-$  (Meeks et al., 1983; Sanz et al., 1995; Mekonnen et al., 2002), while another study reported that  $\text{NH}_4^+$  derived from  $\text{NO}_3^-$  reduction, not  $\text{NO}_3^-$  itself, is ultimately responsible for the suppression of nitrogenase activity (Ramos and Guerrero, 1983). Other studies have reported significantly higher photosynthetic  $\text{O}_2$  evolution (Mishra, 1997) and growth rates (Zulkefli and Hwang, 2020) in  $\text{NH}_4^+$ -grown relative to  $\text{NO}_3^-$ -grown *Dolichospermum*, though in the latter study heterocyst density was significantly higher in  $\text{NH}_4^+$ -grown cultures, suggesting that  $\text{N}_2$  fixation may have been significantly higher. These findings, coupled with differences in growth rate,  $F_v/F_m$ , and  $\text{N}_2$  fixation rate with respect to fixed N type, reveal how  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , and urea differentially alter physiological processes in *Dolichospermum*.

Differential expression of genes associated with diazotrophy both supported observations regarding  $\text{N}_2$  fixation rates and further provided deeper physiological insight regarding this process. Transcripts of *nifB* and *nifD* were both significantly lower in  $\text{NH}_4^+ + \text{P}$ , while the former and latter genes were only significantly downregulated in  $\text{NO}_3^- + \text{P}$  and urea + P, respectively. The gene *nifB* is further associated with Fe-Mo cofactor assemblage (Curatti et al., 2007), while *nifD* encodes for the  $\alpha$ -chain for nitrogenase's Fe-Mo protein (Dos Santos et al., 2012). Significantly lower transcript abundance of *Dolichospermum* sp. 54 *nif* genes in response to  $\text{NH}_4^+$  amendment and/or  $\text{PO}_4^-$  depletion is consistent with previous findings in strains of the same genus. While  $\text{NO}_3^-$  was also reported to have less of an effect on *nif* gene expression than  $\text{NH}_4^+$  in these findings (Helber et al., 1988; Martin-Nieto et al., 1991), transcriptional changes for multiple genes belonging to this cluster in response to the availability of P and multiple fixed N compounds in other *Dolichospermum* taxa have been poorly characterized. It was also shown that when *Dolichospermum* is grown under fixed N-replete or P-deplete conditions, *nifE* and *nifV*, which

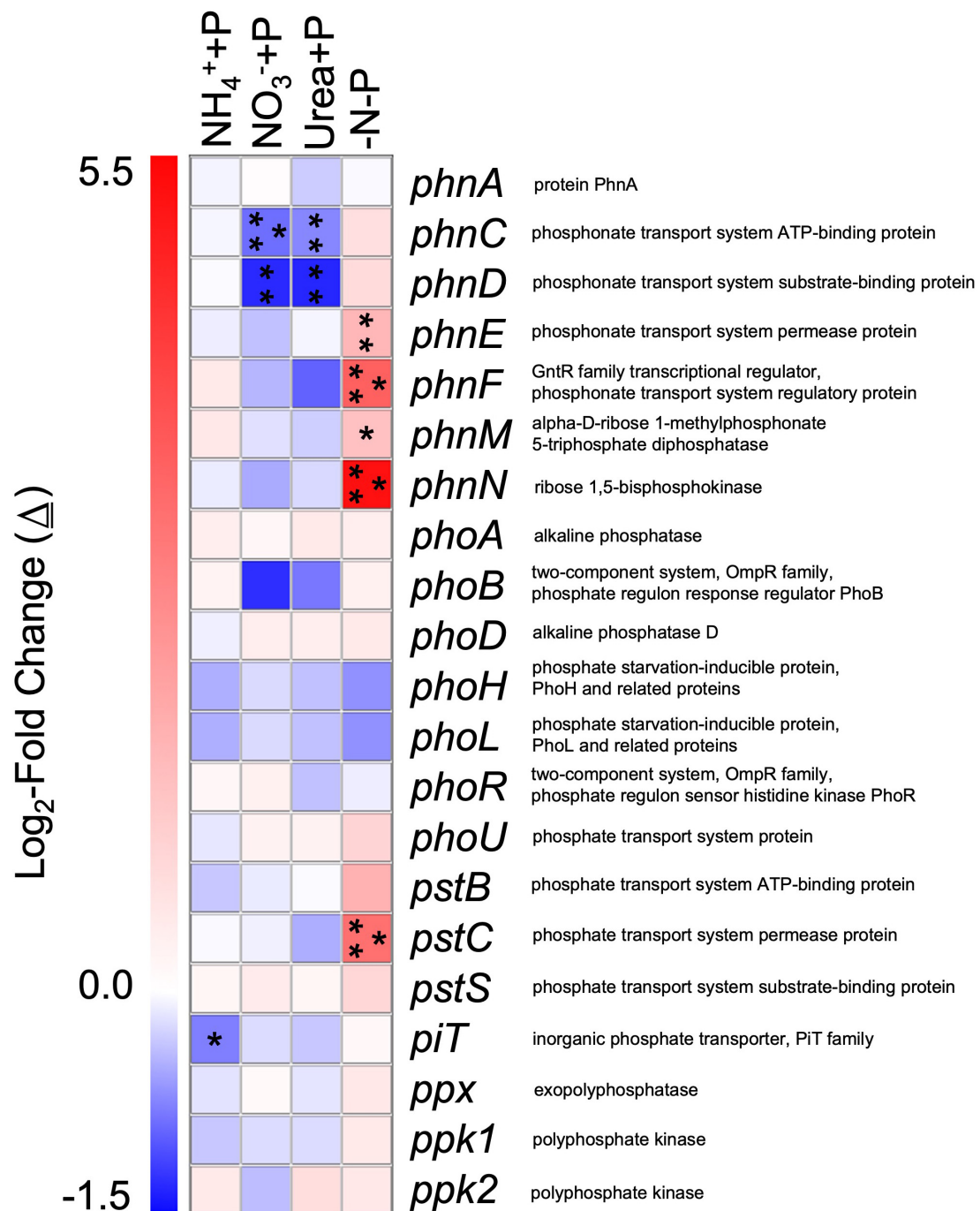


FIGURE 9

Differential expression of gene transcripts associated with phosphorus acquisition and metabolism in experimental groups relative to the control/-N + P. Heatmap representing log<sub>2</sub>-fold changes ( $\Delta$ ) in phosphorus metabolism/acquisition gene expression in experimental groups with their corresponding gene products. Red or blue coloration corresponds to up or down - regulation of genes relative to the control, respectively. Statistical analyses were determined on transcriptomic data post-Sleuth analysis using the Wald test ( $qval < 0.05 = *$ ,  $0.01 = **$ ,  $0.001 = ***$ ).

encode Fe-Mo cofactor proteins (Hawkes et al., 1984; Ugalde et al., 1984), and accessory protein-encoding *nifW* (Nonaka et al., 2019) are all downregulated. Finally, the significant downregulation of *nifD* in NH<sub>4</sub><sup>+</sup>+P and urea + P cultures,

which also exhibited the lowest N<sub>2</sub> fixation rates among treatments, is likely a reliable indicator of repressed N<sub>2</sub> fixation when *Dolichospermum* are actively utilizing either N species.

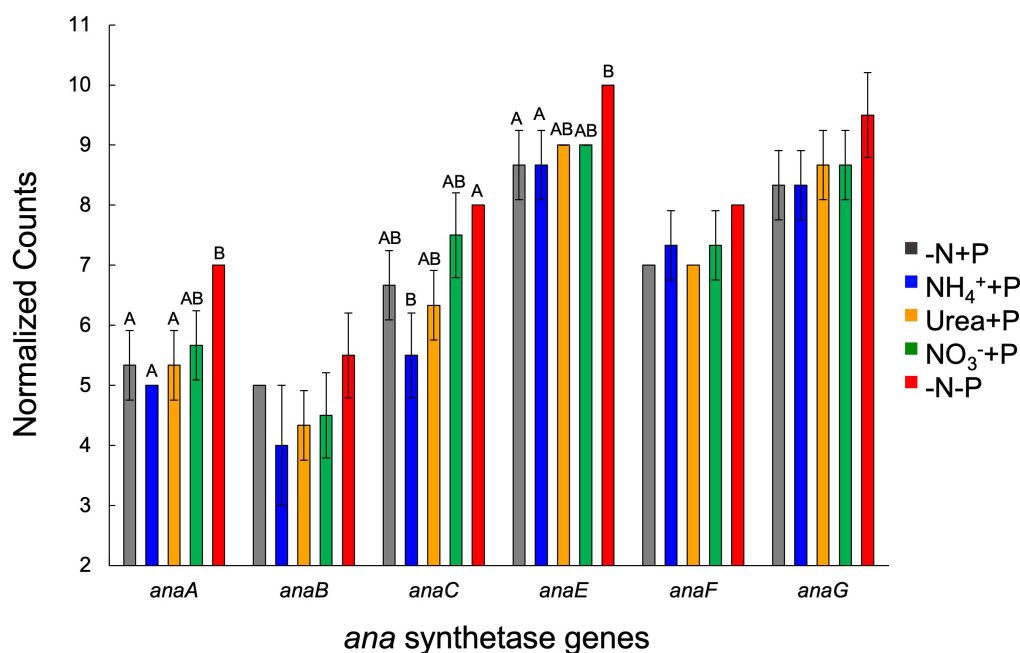


FIGURE 10

Expression (normalized counts) of genes involved in anatoxin synthesis across treatments at the final timepoint (day 9). Error bars represent standard deviation. Letters above bars represent significant differences between experimental groups (Two-way ANOVA; Tukey's HSD *post hoc*;  $p < 0.05$ ).

## Effect of fixed N and P availability on ATX-A production

Anatoxin-a (ATX-A) is a highly potent neurotoxin produced by freshwater cyanobacteria with a broad geographic distribution (Devlin et al., 1977; John et al., 2019; Österholm et al., 2020), yet the factors that control the production of this toxin are largely unknown. Several studies suggest that fixed N (Rapala et al., 1993; Gagnon and Pick, 2012) and/or P (Rapala and Sivonen, 1998; Toporowska et al., 2016) availability regulate ATX-A production. There is also evidence to suggest that ATX-A content in cells is controlled by growth rate, which subsequently regulates the partitioning of toxins into daughter cells (Heath et al., 2016), a concept known as the growth differentiation balance hypothesis (Herms and Mattson, 1992). Significant increases (–N–P) and decreases (NH<sub>4</sub><sup>+</sup> + P) in the cellular ATX-A content of *Dolichospermum* sp. 54 relative to the control (–N + P) and the significant negative correlation between ATX-A cell quota and growth rate suggests that toxin concentration was driven by division rate. However, expression of the anatoxin synthetase gene (*ana*) cluster indicates that factors other than growth rate regulate anatoxin production. Transcripts of *anaA* as well as *anaC–F* were significantly more abundant under P-deplete conditions, indicating cells were actively increasing toxin synthesis in response to nutrient-limited stress and did not simply accumulate more ATX-A

since cells were dividing more slowly. The genes *anaA* and *anaC* encode proteins associated with thioesterase and proline adenylation, respectively, while *anaE* and *anaF* both encode for type I polyketide synthases (Rantala-Ylinen et al., 2011). Of the four genes, *anaC* best predicted variance in ATX-A cellular content among treatments, as both toxin quota and transcript abundance of *anaC* were significantly lower due to NH<sub>4</sub><sup>+</sup> enrichment and increased in response to P depletion. These data, when considered with reports of *anaC* expression exhibiting a significant negative relationship with dissolved carbon-to–Nitrogen (C:N) ratios (Tao et al., 2020), suggests that *anaC* expression and subsequent ATX-A production is highly sensitive to fixed N availability. Ecologically speaking, such an active process could serve as a defense mechanism against grazers (Toporowska et al., 2014; Anderson et al., 2018) or an allelopathic strategy against other phytoplankton (Kearns and Hunter, 2001; Chia et al., 2018, 2019), particularly if anatoxin-producing cyanobacteria are subjected to conditions unsuitable for them to achieve optimal growth rates (Harland et al., 2013; Heath et al., 2016).

The toxin content of *Dolichospermum* sp. 54 also exhibited a positive correlation ( $p < 0.06$ ) with nitrogenase activity, which was driven by experimental treatments with lower growth rates exhibiting higher N<sub>2</sub> fixation rates and cellular toxin content. The manner in which anatoxin synthetase may interact with other biochemical pathways is poorly understood

(Rantala-Ylinen et al., 2011). However, given that different N forms alter growth, photosynthesis, and toxin production in *Dolichospermum* sp. 54 and other cyanobacteria (Harke et al., 2016; Erratt et al., 2018), understanding how nitrogenase activity might directly influence anatoxin production is warranted. To our knowledge, no study has investigated whether nitrogenase activity influences ATX-A synthesis. It is also important to note that other Nostocales taxa have exhibited significantly higher ATX-A concentrations and growth rates when grown on urea rather than  $\text{NH}_4^+$  and  $\text{NO}_3^-$  (Qian et al., 2017; Tao et al., 2020). Urea + P cultures exhibited no significant difference in ATX-A cell quota relative to the control. This suggests that the ability for cyanobacteria to increase anatoxin production when using urea as a fixed N source may be dependent on whether they can fix  $\text{CO}_2$  derived from urease activity (Krausfeldt et al., 2019), as ATX-A is a toxin with a high C:N ratio (Van de Waal et al., 2014). Tao et al. (2020), who observed significantly higher growth rates and ATX-A quotas in the Nostocales taxon *Cuspidothrix issatschenkoi* when cultures were grown on urea relative to other fixed N sources, suggested as much in their discussion. Consistent with our findings, they further reported that  $F_v/F_m$  was significantly lower in urea-grown cultures than those grown on  $\text{NO}_3^-$  (Tao et al., 2020), which further reinforces the notion that reductants from photosynthetic light reactions can be used to reduce  $\text{NO}_3^-$  (Flores et al., 1983, 2005) but not convert urea to  $\text{NH}_4^+$ .

## Global transcriptomic changes in response to fixed N and P availability

The transcriptomes of fixed N-amended cultures exhibited considerable similarity to each other and to the control, while the transcriptomes of  $-\text{N}-\text{P}$  were the most dissimilar from other treatments and exhibited the greatest number of differentially expressed genes relative to the control. In addition, all experimental treatments exhibited a greater number of downregulated genes than upregulated genes relative to  $-\text{N} + \text{P}$  transcriptomes. These findings complement those of previous studies, in which removal of  $\text{NO}_3^-$  or  $\text{NH}_4^+$  in *Dolichospermum* cultures led to a greater number of significantly upregulated rather than downregulated genes associated with heterocyst production as well as stress responses to fixed N deprivation (Ehira and Ohmori, 2006; Flaherty et al., 2011; Mitschke et al., 2011). However, it is important to note that the  $-\text{N}-\text{P}$  treatment was the least different from the control relative to growth,  $F_v/F_m$ ,  $\text{N}_2$  fixation, and ATX-A quota. Thus, it is likely that fixed N and P deprivation significantly influenced components of the *Dolichospermum* transcriptome beyond genes associated with growth, toxin production, and nitrogen assimilation.

## Effects of nutrient availability on N and P assimilation

N form as well as P depletion led to significant differential expression of specific genes associated with N metabolism and  $\text{N}_2$  fixation relative to the control. The downregulation of *amt1*, which encodes for an ammonium transporter, and *cphA*, which encodes for cyanophycin synthetase, in  $\text{NH}_4^+ + \text{P}$  relative to the control, for instance, is consistent with previous findings, as cyanobacteria generally upregulate these genes only when fixed N is scarce (Montesinos et al., 1998; Muro-Pastor et al., 2005; Paz-Yepes et al., 2008). Nutrient availability also significantly affected the expression of genes associated with the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway, a major metabolic process by which  $\text{NH}_4^+$  is assimilated (Wolk et al., 1976). For example, *glnB*, which encodes for the regulatory protein PII (Forchhammer, 2004), was downregulated in the transcriptomes of  $\text{NH}_4^+ + \text{P}$ , which is consistent with previous findings (Tsinoremas et al., 1991; Paz-Yepes et al., 2009). PII activity is essential to the C:N balance in cyanobacteria and increases with increasing levels of 2-oxoglutarate, which is an indicator of the balance between  $\text{CO}_2$  fixation and  $\text{NH}_4^+$  assimilation (Muro-Pastor et al., 2001; Forchhammer, 2004; Valladares et al., 2008). 2-oxoglutarate levels also regulate the activity of NtcA, a global transcriptional regulator for N assimilation pathways (Zhao et al., 2010; Picossi et al., 2014). The activity and levels of NtcA and 2-oxoglutarate, respectively, were not measured in this study. However, *gltD*, which encodes for glutamate synthase (Goss et al., 2001) was significantly downregulated in urea + P relative to the control. As glutamate is produced by glutamate synthase transferring nitrogen from glutamine to 2-oxoglutarate (Walker and van der Donk, 2016), it is highly likely that 2-oxoglutarate levels, as well as NtcA activity, are significantly affected when P-replete *Dolichospermum* sp. 54 are given urea as a fixed N source.

Downregulation of *glnL*, which encodes for nitrogen regulator II (NRII) (Ninfa and Magasanik, 1986), was observed in the transcriptomes of  $\text{NO}_3^- + \text{P}$  and  $-\text{N}-\text{P}$ . Downregulation of *gltD*, which encodes for part of the NADPH-GOGAT (Okuhara et al., 1999), was also observed in the transcriptomes of urea + P. The significant downregulation of *glnL* expression suggests that glutamine synthesis significantly decreased in these treatments (Reitzer and Magasanik, 1985; Ninfa and Magasanik, 1986), and reinforces the notion that a large portion of N derived from  $\text{NO}_3^-/\text{NO}_2^-$  reduction is not incorporated into organic compounds but rather utilized to promote PSII efficiency as oxidants (Flores et al., 2005). This is likely due to *Dolichospermum* requiring considerable amounts of energy to completely reduce  $\text{NO}_3^-$  (Ramos and Guerrero, 1983; Elder and Parker, 1984) and accounts for the significantly slower growth rates of  $\text{NO}_3^-$ -grown cells relative to  $\text{NH}_4^+$ -grown cells. Differential expression



of genes within the GS-GOGAT pathway in different N treatments provides insight regarding how each fixed N compound affected N and C metabolism in *Dolichospermum* sp. 54.

Genes that encode for nitrate (*narB*) and nitrite (*nirA*) reductases (Cai and Wolk, 1997; Herrero and Flores, 2018) also exhibited significant upregulation and no significant difference in expression, respectively, in  $\text{NO}_3^- + \text{P}$  and  $-\text{N}-\text{P}$  cultures. This suggests that while  $\text{NO}_3^-$  was being reduced to  $\text{NO}_2^-$  (*narB*),  $\text{NO}_2^-$  reduction to  $\text{NH}_4^+$  (*nirA*) was occurring at a relatively lower rate, which likely reflects  $\text{NO}_3^- + \text{P}$  cultures growing at a significantly slower rate than  $\text{NH}_4^+ + \text{P}$  cultures. Regardless, upregulation of *narB* likely reflects the significantly higher  $F_v/F_m$  and the significant enrichment of gene categories associated with the photosynthetic apparatus in  $\text{NO}_3^- + \text{P}$  cultures than that of the control, as  $\text{NO}_3^-$  reduction enhances cyanobacterial PSII efficiency as a Hill reagent (Serrano et al., 1981; Flores et al., 1983, 2005). While enhanced nitrate reductase activity in another *Dolichospermum* strain was reported to be essential in inhibiting nitrogenase activity (Martin-Nieto et al., 1991), that was not the case for  $\text{NO}_3^- + \text{P}$  cultures of *Dolichospermum* sp. 54, which exhibited significantly higher transcript abundance of *narB* and no significant change in  $\text{N}_2$  fixation relative to the control. This suggests that other factors, such as the full reduction of  $\text{NO}_3^-$  to  $\text{NH}_4^+$ , are necessary to inhibit nitrogenase activity in members of this genus to a degree comparable to *Dolichospermum* amended with  $\text{NH}_4^+$  and urea.

Gene categories associated with  $\text{PO}_4^{3-}$  transport were significantly upregulated in the transcriptomes of  $-\text{N}-\text{P}$  relative to the control, a response that is consistent with the effect of P-depletion on other Nostocales taxa (Teikari et al., 2015; Dong et al., 2019). The  $-\text{N}-\text{P}$  treatment, which exhibited significantly higher APA activity relative to the control, was the only treatment to exhibit significant upregulation of several *phn* genes and *pstC*. The *phn* gene cluster encodes for proteins involved in the transport and metabolism of phosphonates under P-deplete conditions and/or components of the carbon-phosphorus lyase, while *pstC* encodes for a high affinity  $\text{PO}_4^{3-}$  transporter (Adams et al., 2008; Voß et al., 2013). Collectively, differentially expressed genes associated with P transport and assimilation are consistent with prior studies of gene markers of P-limitation in cyanobacteria (Harke et al., 2012; Harke and Gobler, 2013; Lu et al., 2019) and suggests they are important in supporting *Dolichospermum* blooms when dissolved inorganic phosphorus is scarce.

## Conclusion

In summary, fixed N-replete *Dolichospermum* sp. 54 cultures exhibited significantly enhanced growth ( $\text{NH}_4^+$ , urea,  $\text{NO}_3^-$ ) and photosynthetic efficiency ( $\text{NH}_4^+$  and  $\text{NO}_3^-$ ) relative

to those grown under fixed N-limiting conditions.  $\text{NH}_4^+$ -grown cultures also exhibited significantly higher and lower growth and  $\text{N}_2$  fixation rates, respectively, relative to  $\text{NO}_3^-$ -grown cultures. Cultures also exhibited significant differences in growth,  $F_v/F_m$ ,  $\text{N}_2$  fixation, ATX-A production, and genome expression depending on whether they were amended with  $\text{NH}_4^+$ , urea,  $\text{NO}_3^-$ , or deprived of both fixed N and P, reinforcing the notion that *Dolichospermum* and likely other toxin-producing Nostocales taxa exhibit highly specific preferences for different fixed N species. These findings also support the growing consensus that diazotrophic cyanobacteria exhibit fixed N-limitation similar to non-diazotrophic CHAB taxa such as *Microcystis* with respect to growth rate, as well as P-limitation, and prefer certain fixed N species under P-replete conditions. From a managerial perspective, if fixed N inputs are not controlled in eutrophic systems where *Dolichospermum* spp. occur, blooms of these and other Nostocales taxa may be of greater biomass and subsequently have higher toxin concentrations.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

## Author contributions

BK and JJ performed the culture experiment and analyzed growth rate, ATX-A,  $\text{N}_2$  fixation, nutrient, and  $F_v/F_m$  data. BK graphed the data and drafted the manuscript. JJ extracted RNA. MH and DN processed transcriptomic data post-sequencing. CG provided the funding and technical resources necessary to perform experiments and performed the analyses. All authors contributed to data analysis and study design.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.955032/full#supplementary-material>

### SUPPLEMENTARY FIGURE 1

Cell densities for all treatments during the experiment, from day 1 to the final time point (day 9). Error bars represent standard deviation.

### SUPPLEMENTARY FIGURE 2

Photosystem II photosynthetic efficiency ( $F_v/F_m$ ) for all treatments during the experiment. Error bars represent standard deviation. Red arrows represent days when fixed N species ( $\text{NH}_4\text{Cl}$ , Urea, and  $\text{NaNO}_3$ ) were added to fixed N-replete treatments.

### SUPPLEMENTARY FIGURE 3

Alkaline phosphatase activity ( $\text{nmol mL}^{-1} \text{ hr}^{-1}$ ) as a function of volume and time in treatments deprived on nitrogen and either with or without phosphorus on days 1, 3, 5, and 9. Error bars represent standard error. Number of asterisks correspond to degree of significance ( $p < 0.05 = *$ ,  $0.01 = **$ ,  $0.001 = ***$ ), determined via Student's paired  $t$ -test.

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