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Mutual Dependence of Nitrogen and Phosphorus as Key Nutrient Elements: One Facilitates *Dolichospermum flos-aquae* to Overcome the Limitations of the Other

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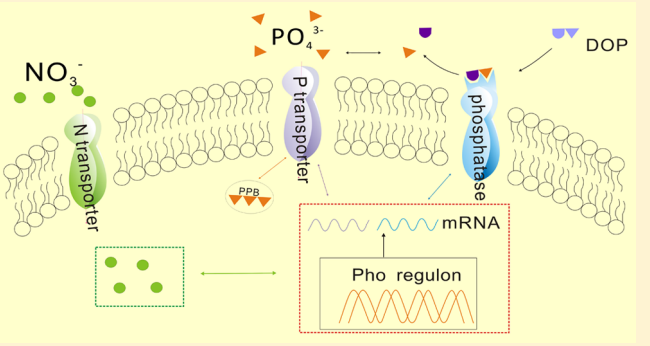
Supporting Information

ABSTRACT: *Dolichospermum flos-aquae* (formerly *Anabaena flos-aquae*) is a diazotrophic cyanobacterium causing harmful blooms worldwide, which is partly attributed to its capacity to compete for nitrogen (N) and phosphorus (P). Preventing the blooms by reducing P alone or both N and P has caused debate. To test the effects alone and together on the growth of cyanobacteria, we performed culture experiments in different eutrophication scenarios. N_2 fixation in terms of heterocyst density, nitrogenase activity and *nifH* expression increased significantly in P-replete cultures, suggesting that P enrichment facilitates N_2 fixation. Correspondingly, the expression of genes involved in P uptake, e.g., those involved in P-transport (*pstS*) and the hydrolysis of phosphomonoesters (*phoD*), was upregulated in P-deficient cultures. Interestingly, N addition enhanced not only the expression of these genes but also polyphosphate formation and alkaline phosphatase activity in P-deficient cultures relative to the P-replete cultures, as evidenced by qualitative (enzyme-labeled fluorescence) and quantitative (fluorogenic spectrophotometry) measurements. Furthermore, after N addition, cell activity and growth increased in the P-deficient cultures, underscoring the risk of N enrichment in P-limited systems. The eco-physiological responses shown here help further our understanding of the mechanism of N and P colimitation and underscore the importance of dual N and P reduction in controlling cyanobacterial blooms.



1. INTRODUCTION

Eutrophication and cyanobacterial blooms pose risks to human and ecosystem health and have been attributed to overenrichment with phosphorus (P) and nitrogen (N) in water bodies.[1] Dual N and P reduction has become central to water management because N and P colimitation of primary productivity has been shown to be a potentially important process in freshwater environments.[2] However, an argument against combined N and P control is that reducing only P can successfully curb lake eutrophication, while it cannot be controlled by reducing N input because N_2 fixation can alleviate N deficits, making N control irrelevant to eutrophication management.[3−5] On the other hand, this behavior has been shown not to be true for some eutrophic systems where N was also important for the control of eutrophication.[6−8]

The biochemical coupling of N and P on the cellular level remains unclear. Liebig's Law of the Minimum has been used to argue against nutrient colimitation of growth.[9] However, in many cases, N and P have been shown to affect the growth rate simultaneously rather than sequentially.[10,11] Colimitation of N and P is biochemically independent.[12] In contrast, others thought that N and P were biochemically dependent, assuming that P quota enhances N uptake.[11] An investigation of the combined effects of N and P on phytoplankton growth at physiological and ecological levels would clarify this issue.

The growth of *Dolichospermum* spp. (formerly *Anagbaena* spp.), bloom-forming, cyanotoxin-producing cyanobacteria, is more likely to be limited by P rather than N since they can fix atmospheric N_2 into biologically available NH_3.[3,13] Furthermore, N_2 fixation, a metabolically expensive process, is controlled by P availability.[6] Cyanobacteria have developed several strategies in response to low inorganic P (P_i) supply, thereby, reducing their P-requirements,[14] using cellular phosphate and/or enhancing P_i acquisition by synthesizing phosphate transporters[15,16] and increasing the affinity and rate of P_i uptake.[17] The P-specific transport (Pst) system comprises a periplasmic P_i-binding protein (PstS) and sensitively responds to P deficiency.[18] The ability to use the dissolved organic phosphorus (DOP) pool, mediated by alkaline phosphatase

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Table 1. Amplification Primers used for qPCR and PCR

gene	protein	amplification primers 5′−3′	PCR product (bp)	efficiency (%)	ref
phoD	alkaline phosphatase	(F) GATTACCGAAACCGCTATGC	156	103.1	this study
		(R) CTCAAATGCTTGCCGACTCT			
nifH	nitrogenase iron protein	(F) TGGTCCAGAACCCGGTGTAG	75	102.7	Short and Zehr[50]
		(R) GCACCGTTTTCTTCTAGGAAGTTAA			
pstS	phosphate-binding periplasmic protein	(F) AAACTCTCAACGGAGCAGGA	129	97.8	this study
		(R) GCCGAATACCACCACCACTA			
rpoC1	RNA polymerase	(F) GCCCAATGGTGCAGTTAGAT	127	105.5	this study
		(R) ACGATAATTTCCGGTGCAAG			
16S	ribosomal RNA	(F) AAAACGGAGAGTTTGATCCT	1489	NA[a]	this study
		(R) AAAGGAGGTGATCCAGCCAC			
phoD	alkaline phosphatase	(F) GTGAATATGCAATTAATAAATCGCA	1575	NA	this study
		(R) TCACGCTGTTTTTGCGC			
pstS	phosphate-binding periplasmic protein	(F) ATGAATTTTTCCACCACCGT	1044	NA	this study
		(R) CTATGGTTTGACGTTGCTATTGA			

[a]Not applicable.

(APase), is an additional strategy for P acquisition. The three most important phosphatase genes (*phoX*, *phoA*, and *phoD*) have been recognized within marine microorganisms.[19] Protein structure analysis revealed that *phoD* encodes APase in *Anabaena*.[20] *Anabaena* sp. always produces extracellular APase, as evidenced by enzyme-labeled fluorescence (ELF) in the natural waters.[21] Another strategy used by microbes to survive P_i-deficiency is that they store P_i in the form of polyphosphate (poly-P) in cells when P is abundant, followed by the breakdown of poly-P stores upon P stress.[22]

This study investigated whether N also plays a role in P scavenging as P does in N utilization. We measured the transcript levels of genes involved in P_i assimilation (*pstS*), DOP hydrolysis (*phoD*), and N_2 fixation (*nifH*); the quantity and quality of APase and poly-P body (PPB) formation in monocultures of *D. flos-aquae* FACHB 245 under N- or P-replete and N- or P-deficient conditions. These experiments provided insight into the molecular and eco-physiological responses of N_2-fixing cyanobacteria to varying exogenous N and P concentrations to test the hypothesis that N and P metabolism is biochemically dependent in cyanobacteria and therefore underscores the need for dual N and P reduction in controlling eutrophication and cyanobacterial blooms. The laboratory-scale experiments help to develop an understanding of microbial nutrient cycling in natural environments.

2. MATERIALS AND METHODS

2.1. Experimental Design. *D. flos-aquae* FACHB 245 was obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology (FACHB-collection, Wuhan, China). Prior to experiments, *D. flos-aquae* cells were harvested in the logarithmic growth period by centrifugation (4000 rpm, 8 min), rinsed 3 times with N- and P-free BG11 medium[23] and then inoculated in medium for 48 h to exhaust nutrients stored in cells. Two experiments probing the responses of gene expression and N_2 fixation to varying concentrations of P and N were performed separately.

2.1.1. Experiment on Responses of Gene Expression to Varying Concentrations of P and N. At the onset of the experiment, monocultures of *D. flos-aquae* were exposed to two P concentrations (−P, 0.05 mg L^{-1}; +P, 2 mg L^{-1}, mimicking P-deficient and P-replete status, respectively) with 0.5 mg NO_3^--N L^{-1} (mimicking N-deficient conditions) in triplicate. The different nutrient treatments were obtained by modifying the nitrate ($NaNO_3$) and phosphate (KH_2PO_4) concentrations in regular BG11 medium. On day 9, we added nitrate ($NaNO_3$) to the treatments (final concentration, 2 mg L^{-1}). The experiment was conducted in 500 mL Erlenmeyer flasks filled with 200 mL of medium containing *D. flos-aquae* and lasted 16 days. The experimental units were maintained in conditions suitable for cyanobacterial growth: constant photoperiod (12 h of light: 12 h of dark), 30 μmol photons m^{-2} s^{-1} irradiance, and 25 °C. Subsamples were collected every 2 days for cell density, chemical, and enzymatic analysis and quantitative real-time PCR (qPCR) to estimate the expression of *phoD*, *pstS*, and *nifH* genes.

2.1.2. Determining Responses of N_2-Fixation to Varying Concentrations of P and N. Monocultures of *D. flos-aquae* were exposed to different P concentrations (0, 0.05, 0.5, and 2 mg L^{-1}) at 0.5 mg NO_3^--N L^{-1} (mimicking N-deficient conditions) and different N concentrations (0, 0.5, 2, and 10 mg L^{-1}) at 0.05 mg L^{-1} P in triplicate. The experiment lasted 8 days. Subsamples were examined for heterocyst density and nitrogenase activity every 2 days.

2.2. Biological, Chemical, and Biochemical Analysis. Samples for cell and heterocyst density estimation were preserved with Lugol's solution and counted with an Olympus BX 41 microscope (Olympus Corporation, Japan). Chlorophyll fluorescence was examined by Phyto-PAM (Walz, Effeltrich), the photochemical efficiency of photosystem II (F_v/F_m) was determined according to Ting and Owens,[24] and the values of maximum relative electron transport rate ($rETR_{max}$) were plotted *vs* irradiance to give the corresponding rapid light curve, which was automatically recorded by preset software in the Phyto-PAM.[25]

Samples were processed by filtering through 0.45 μm (Millipore) filters, and an analysis of soluble reactive phosphorus (SRP) and nitrate (NO_3^--N) concentrations by a standard method[26,27] was then performed. Alkaline phosphatase activity (APA) was quantified by fluorometric assay.[28] Nitrogenase activity was analyzed by the acetylene reduction assay.[29] Qualitative levels of extracellular APases from *D. flos-aquae* were detected using ELF 97 phosphate (Molecular Probes) on the basis of the protocol proposed by Štrojsová et al.[21]

PPBs are linear polymers of several to several hundred phosphate groups and are found extensively in microbial cells;[30] many microorganisms and even cyanobacteria can excessively

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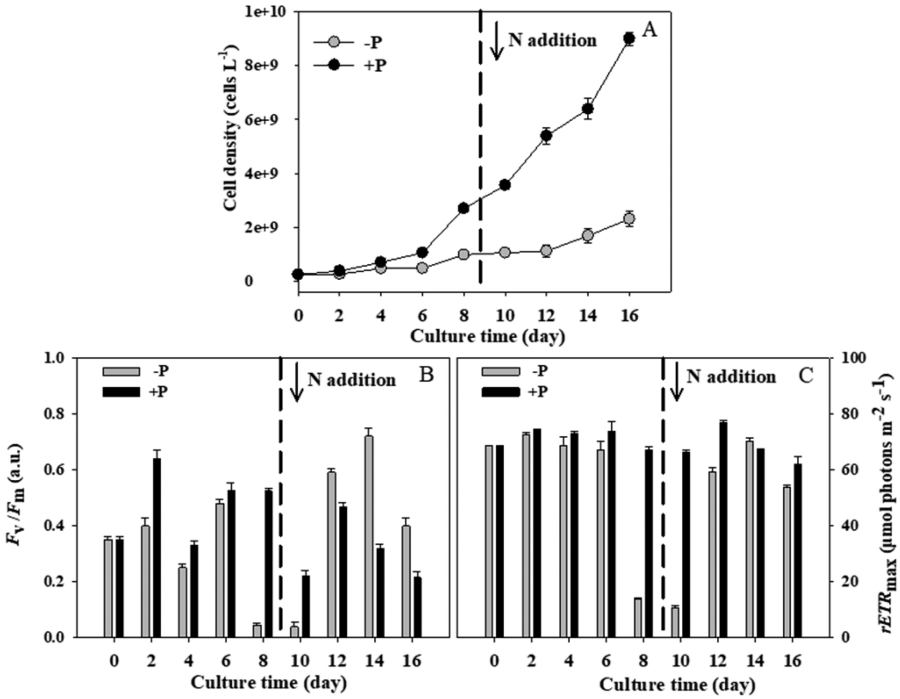

Figure 1. Time series of cell density (A), maximum photochemical efficiency (F_v/F_m) (B), and maximum relative electron transport rate ($rETR_{max}$) (C) in P-deficient (−P) and P-replete (+P) cultures before and after N addition. Error bars represent standard deviation between biological replicates.

absorb P_i and store it in cells in the form of PPBs.[22] Fluorescence staining by 4′,6-diamidino-2-phenylindole (DAPI) was used to examine *D. flos-aquae* for PPBs with epifluorescence microscopy (Olympus BX51FL) with UV excitation according to the procedure of Bar-Yosef et al.[31]

2.3. Molecular Analysis. *D. flos-aquae* cells from culture samples were concentrated and pelleted by centrifugation.[32] Total RNA was extracted for analysis by using the Bioline ISOLATE II RNA Mini kit, following the manufacturer's protocol, and we used DNase Max Kits (MoBio, USA) to remove the total DNA. The purity and yield of RNA were assessed with a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific, USA). cDNA was reverse transcribed from RNA using the Promega GoScript Reverse Transcription System.

The primers for *pstS*, *phoD*, and *rpoC1* (housekeeping gene) amplification were identified from CyanoBase (http://genome.microbedb.jp/cyanobase) and designed using Primer-BLAST (Supplemental Methods, Table 1). Moreover, we ran BLASTN to compare primers against other genomes to ensure their validity and specificity.

Triplicate reactions were carried out with SYBR Green PCR Master Mix, and the reaction system (20 μL) contained 10 μL of 2 × Mix, 0.4 μM forward and reverse primers and 1 μL of 1:10 diluted cDNA. qPCR was conducted in an Applied Biosystems 7900 Real-Time PCR System (Applied Biosystems, USA), and the qPCR program for target genes was 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s, and followed by 1 step of 72 °C for 7 min. To normalize the qPCR data, we chose the RNA polymerase gene *rpoC1* as a suitable housekeeping gene for the analysis of gene expression levels. Fold changes in gene transcript levels were calculated as $2^{-\Delta\Delta Ct}$. For the $2^{-\Delta\Delta Ct}$ equation to be valid, the amplification efficiency values of target genes and housekeeping gene must be approximately equal (less than 0.1). We estimated the transcript abundances of *phoD*, *pstS*, and *nifH* within cDNA samples based on the known copy numbers of linearized plasmids of *phoD*, *pstS*, and *nifH* gene clones. Finally, the values were calculated as gene transcript abundances (copies mL^{-1} culture). The amplification efficiencies of primer pairs were 97.8−105.5% ($R^2 > 0.99$) (Table 1).

2.4. Statistical Analysis. Both two-tailed Pearson's correlation tests and analyses of variance were performed with SPSS 18.0 (Chicago, USA) to examine the relationships or differences between parameters, and differences were considered statistically significant at $P < 0.05$ or $P < 0.01$.

3. RESULTS

3.1. Experiment on Responses of Gene Expression to Varying Concentrations of P and N. *3.1.1. Culture Growth.* The biomass of *D. flos-aquae* was represented by the cell density (Figure 1A), which increased slightly, from 2.45 × 10^8 (day 0) to 9.79 × 10^8 cells L^{-1}, in the −P treatment and increased significantly, from 2.45 × 10^8 to 2.69 × 10^9 cells L^{-1}, in the +P treatment. After N addition, the cell density continuously increased to 2.31 × 10^9 and 9.0 × 10^9 cells L^{-1} in the −P and +P treatments separately.

3.1.2. Photosynthetic Activity. Photosynthetic activity was represented by chlorophyll fluorescence. Under N-deficient conditions, samples produced values of F_v/F_m that ranged between 0.045 ± 0.007 and 0.48 ± 0.014 in the −P culture, which are lower than those in the +P treatment (0.330 ± 0.017 to 0.640 ± 0.028). After N addition, they increased significantly (0.720 ± 0.030) in the −P culture, even higher than those in the +P treatment (Figure 1B). Before N addition, the values of $rETR_{max}$ ranged from 13.65 ± 0.50 to 72.65 ± 0.64 and 67.00 ± 1.27 to 74.10 ± 0.28 μmol photons m^{-2} s^{-1} in the −P and +P cultures, respectively. Two days after N addition, $rETR_{max}$ increased sharply, and its value remained high (53.70−70.25

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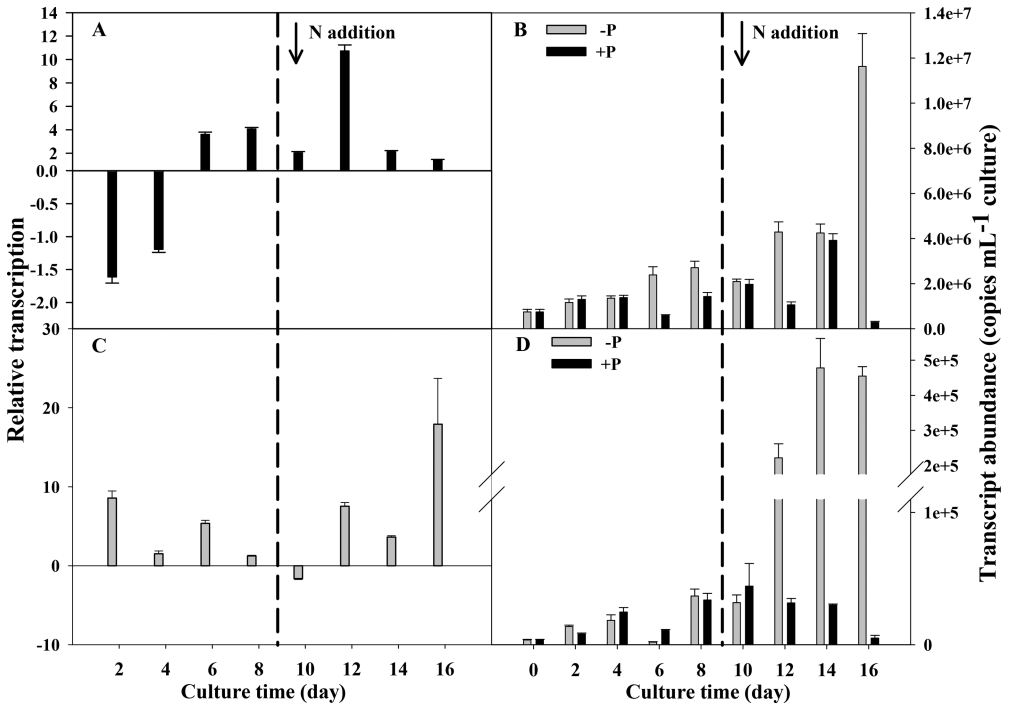

Figure 2. Expression levels of *pstS* (A) and *phoD* (C) genes in the low phosphorus (−P) cultures relative to those of the high phosphorus (+P) cultures and a time series of *pstS* (B) and *phoD* (D) gene transcript abundance with low phosphorus (−P) and high phosphorus (+P) concentrations in cultures of *Dolichospermum flos-aquae* FACHB 245.

Table 2. Pearson's Coefficients Describing the Correlation between Environmental Parameters and the Transcript Abundances of *phoD*, *pstS*, and *nifH*[a]

parameters	F_v/F_m	$rETR_{max}$	NO_3^--N	SRP	NO_3^--N/SRP	heterocyst
transcript abundance of *phoD*	0.082	−0.101	0.572*	−0.539*	0.620*	0.169
transcript abundance of *pstS*	0.019	−0.192	0.549*	−0.614*	0.666**	−0.080
transcript abundance of *nifH*	0.077	0.237	−0.230	0.723**	−0.624**	0.515*

[a]Significance at **α = 0.01 level; *α = 0.05 level.

μmol photons m^{-2} s^{-1}) in the −P cultures. In contrast, +P treatment averaged an $rETR_{max}$ of 68.01 ± 6.37 μmol photons m^{-2} s^{-1} and did not change significantly (Figure 1C).

3.1.3. Sequence Analysis. Phylogenetic analyses indicated that the 16S rRNA sequence of *D. flos-aquae* FACHB 245 shared high homology with those of *Anabaena variabilis* and *Anabaena* sp. PCC 7120 (Figure S1). Sequence analysis of *D. flos-aquae* revealed that *phoD*, *pstS*, and *nifH* were present and conserved. Phylogenetic analysis of *pstS* and *phoD* sequences among *D. flos-aquae* and model cyanobacteria showed that the *D. flos-aquae* sequences formed distinct clusters (Figures S2, S3). Based on cluster analysis, *D. flos-aquae* appeared most closely related to heterocystous cyanobacteria in terms of the phosphate-binding periplasmic protein PstS and the APase PhoD (Figures S2, S3).

3.1.4. Responses to Varying Concentrations of P and N. The *pstS* (all0911; phosphate-binding periplasmic protein gene) gene was amplified by our primers from clones of *D. flos-aquae*. As revealed in Figure 2A, relative to its value with the +P treatment, the expression of *pstS* in the −P treatment was initially significantly downregulated (1.4-fold, days 2−4) but significantly upregulated (2.8-fold) later (days 6−8). After N addition, *pstS* in the −P treatment was noticeably upregulated (10.7-fold) on day 12.

The transcript abundance of *pstS* in −P cultures increased gradually from 7.5×10^5 (day 0) to 2.7×10^6 copies mL^{-1} culture but varied slightly in the +P treatment (from 7.5×10^5 (day 0) to 1.4×10^6 copies mL^{-1} culture). After N addition, transcript levels in the −P cultures increased significantly, peaking on day 16 at 1.2×10^7 copies mL^{-1} culture. Transcript levels in the +P cultures showed a general increasing trend until day 14 and then decreased significantly (Figure 2B). Additionally, the correlation between the transcript abundance of *pstS* and the ambient SRP concentration was significantly negative ($r = -0.614$, $P < 0.01$), but the correlation between the transcript abundance of *pstS* and the ambient NO_3^--N concentration showed a positive relationship ($r = 0.549$, $P < 0.05$) (Table 2).

Before N addition, no PPB-containing cells were detected in the −P cultures from day 2 to day 6, and 6.86% of cells had PPBs on day 8 (Figure 4). After N addition, the PPB-containing cells gradually disappeared. In contrast, the percentage of PPB-containing cells in the +P cultures was very low (less than 0.3%) before N addition, while the percentage and amount of PPB-containing cells (Figure 3) increased significantly and then reached their highest values (11.08% and 3.95×10^8 cells L^{-1}) on day 10 after N addition, with an ambient SRP concentration of 0.76 mg L^{-1}. Their values then decreased gradually over time.

The transcript abundance of *phoD* ranged from 1.8×10^3 to 3.7×10^4 and 3.5×10^3 to 3.4×10^4 copies mL^{-1} culture in −P and +P cultures, respectively. However, transcript levels in −P

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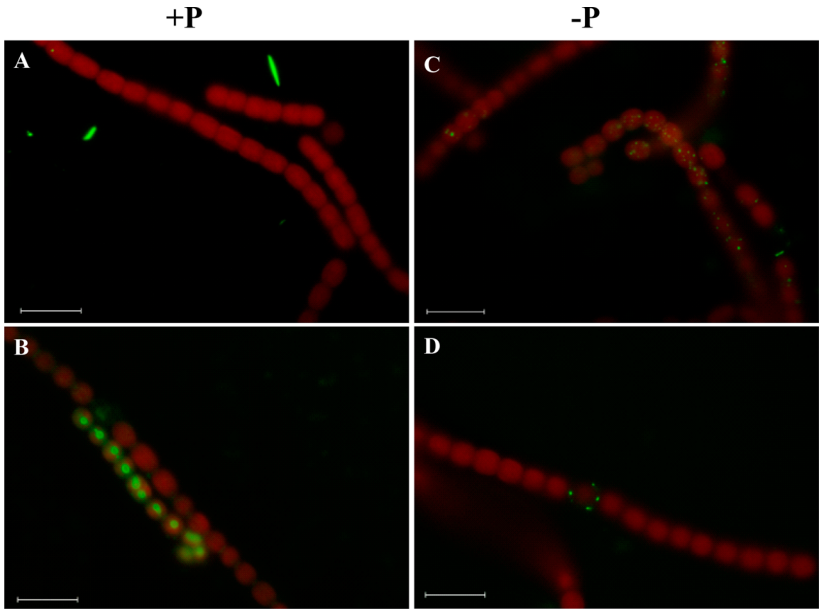

Figure 3. Microscopic observations of *D. flos-aquae* FACHB 245. Cells grown in P-replete (+P) and P-deficient (−P) medium were treated with ELF reagent and DAPI (for extracellular phosphatase and polyphosphate body (PPB) detection, respectively). Scale bars indicate 10 μm. (A) Cells grown in +P cultures and treated with ELF reagent. No green fluorescence associated with cells due to ELF was observed. (B) Cells grown in +P cultures and treated with DAPI. Green fluorescence associated with cells due to DAPI was observed. (C) Cells grown in −P cultures and treated with ELF reagent. Green fluorescence associated with cells due to ELF was observed. (D) Cells grown in −P cultures and treated with ELF reagent. Green fluorescence associated with heterocysts due to ELF was observed.

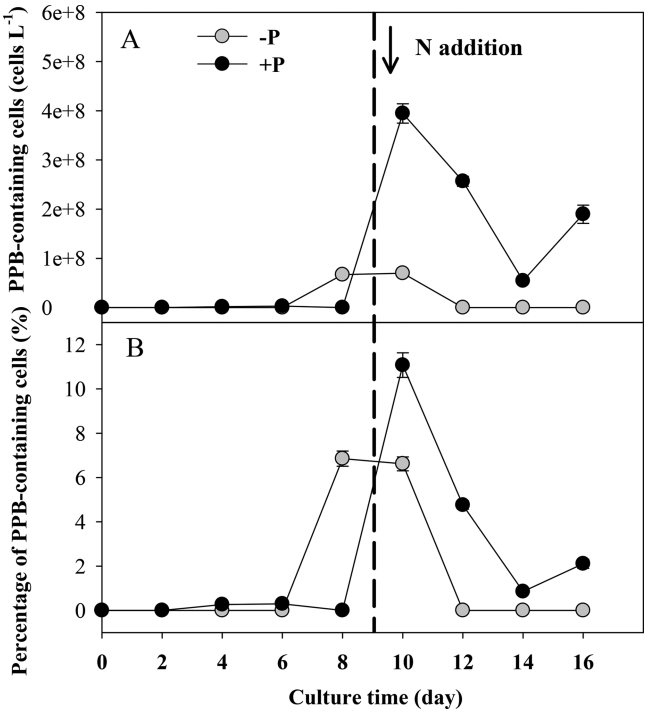

Figure 4. Time series of density (A) and percentage (B) of PPB-containing cells in P-deficient (−P) and P-replete (+P) cultures before and after N addition.

cultures increased significantly, from 3.2×10^4 (day 0) to 4.8×10^5 copies mL^{-1} culture, while transcript levels in the +P cultures displayed no significant changes over time (Figure 2D). Meanwhile, the *phoD* gene was amplified by our primers in clones of *D. flos-aquae* and displayed higher expression in the −P cultures, mainly after N addition. Additionally, relative to its level in the +P cultures, *phoD* was upregulated 1.2- to 8.6-fold before N addition. After day 8, the expression was significantly upregulated (to 17.9-fold, Figure 2C). Additionally, there was a significantly positive relationship between the transcript abundances of *pstS* and *phoD* ($r = 0.7$, $P < 0.01$, $n = 16$).

ELF detection showed that *D. flos-aquae* in the −P cultures secreted an extracellular APase (Figure 3C). Heterocysts, when present, were always ELF-labeled (Figure 3D). After N addition, ELF-labeled cells were detected in cultures of −P and +P treatments, especially in the last sample of the experiment. Quantitatively, APA negatively related to SRP concentrations ($r = 0.8$, $P < 0.01$, $n = 16$).

3.2. Responses of N_2-Fixation to Varying Concentrations of P and N. The density of heterocysts increased with time in some treatments (0, 0.5, and 2 mg L^{-1} NO_3^--N L^{-1}) and decreased gradually with increasing N concentration, varying from 4.74×10^7 to 0 cells L^{-1} (Figure 5A). Nitrogenase activities showed a trend similar to that of heterocyst density, ranging from 0 to 3.05 nmol C_2H_4 mL^{-1} h^{-1} (Figure 5C). N-free treatment resulted in the highest values for both parameters. In the scenarios of varying P with relatively low N concentrations (0.05 mg L^{-1}), nitrogenase activities and heterocyst density in the treatments with high P concentrations were remarkably higher than those in treatments with low P concentrations ($P < 0.05$), especially at the end of the experiment (day 8; Figure 5B, D). As shown in Table 2, the transcript abundance of *nifH* in the cultures of *D. flos-aquae* was not only significantly and positively related to the amount of heterocysts ($r = 0.515$, $P < 0.05$) and ambient SRP concentration ($r = 0.723$, $P < 0.01$) but also negatively related to the NO_3^--N/SRP ratio ($r = -0.624$, $P < 0.01$).

4. DISCUSSION

Both concentrations and ratios of N and P play important roles in controlling the development of *Anabaena* blooms.[33] Despite

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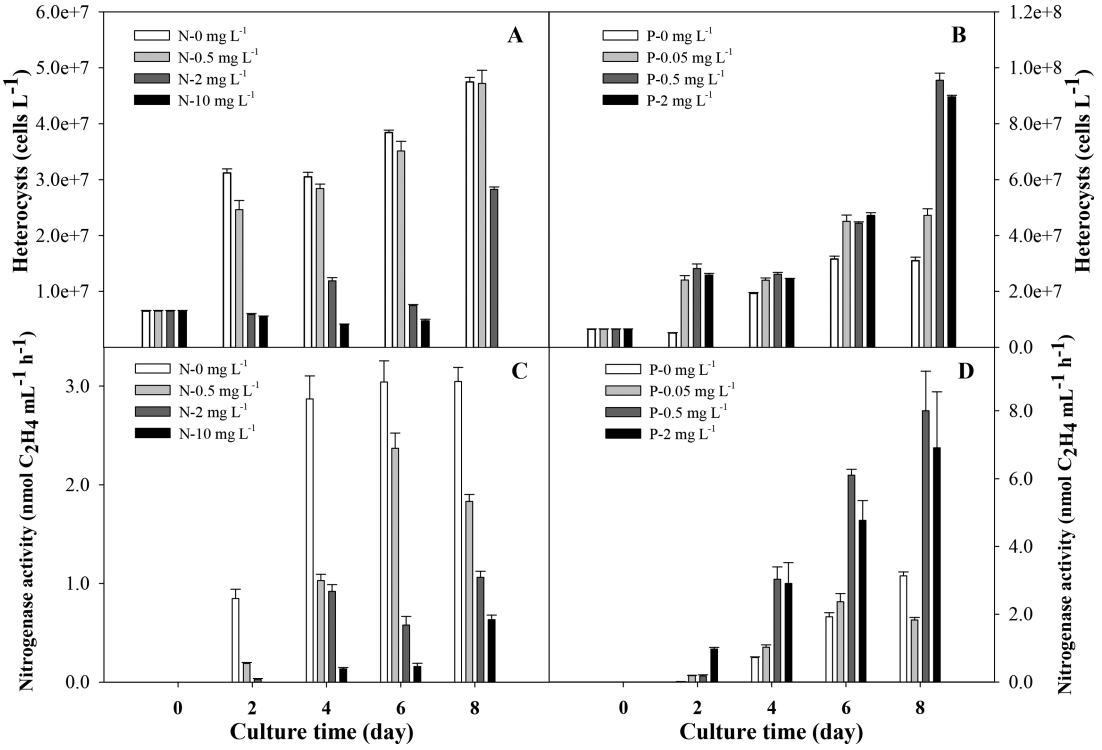

Figure 5. Time series of heterocyst density (A, B) and nitrogenase activity (C, D) with varying N and P concentrations. Plots A and C represent cultures grown with varying N concentrations but at the same P concentrations, while plots B and D represent cultures grown with varying P concentrations but at the same N concentrations.

the sequencing of diazotrophic cyanobacterium genomes over past decades,[34] the molecular response to P or N limitation and their mutual influences are largely unknown. In the present study, the expression of the genes involved in N_2 fixation (*nifH*), P transport (*pstS*), and the hydrolysis of phosphomonoesters (*phoD*) in *D. flos-aquae* was regulated by not only their target nutrient (external inorganic N or P concentrations) but also the other nontarget nutrient. Expression of *nifH* showed a significantly negative relationship with NO_3^-/SRP ratio, while *phoD* and *pstS* significantly responded in a positive manner. N addition greatly increased the expression of both *phoD* and *pstS*, suggesting that N availability may enhance the ability of *D. flos-aquae* to overcome P limitation and *vice versa*.

4.1. Response to Varying N Concentrations and the Effects of P Enrichment. N_2 fixation provides cyanobacteria with a vital competitive advantage during N limitation.[35] The generally accepted fact that heterocyst density is a proxy for N_2 fixation is supported by our study of *D. flos-aquae*.[36] Nitrogenase activity and heterocyst density both increased gradually with decreasing N concentration. This pattern was attributed to energetic costs associated with N utilization by phytoplankton, with NH_4^+ being the most energetically favorable N source and N_2 being the least.[37] The synthesis of nitrogenase and the formation of new heterocysts are extremely energy intensive.[6] Therefore, P may affect N_2 fixation because it is an essential nutrient in energy transport for all organisms. In this study, the expression of *nifH* in the high P treatment was significantly higher, indicating that P played an important role in N_2 fixation. *D. flos-aquae* (Lyngb.) Bréb grows at its maximum rate in P-sufficient combined N-free medium.[38] Additionally, ELF-labeling of heterocysts in the filaments of *D. flos-aquae* suggested it suffered from P limitation earlier than vegetative cells, and it began to excrete extracellular phosphatase (Figure 3D). Similar results were found in monocultures of *Dolichospermum* and *Trichodesmium* by Yema et al.[39] and Orchard et al.,[15] respectively. Indeed, many field studies showed that low P bioavailability constrained N_2 fixation rates of marine and freshwater diazotrophs, while P enrichment increased these rates.[18,40,41] Numerous observations supported N_2 fixation requiring higher P availability, and this requirement possibly being related to increased ATP demands for N_2 fixation[42] and/or increased of enzymes that contain P in their structure synthesis in heterocysts.[43] These findings indicated the dependence of N_2-fixation on P availability and further underscored the risk of P enrichment in N-limited systems as was also proposed by Yema et al.[39]

4.2. Response to Varying P Concentrations and the Effects of N Addition. Molecular and eco-physiological levels indicate that cyanobacteria adapt very well to conditions of P deficiency by increasing the rate and affinity of P uptake[18,44] and by synthesizing alkaline phosphates to hydrolyze DOP.[32] Our results supported previous studies of *D. flos-aquae*. P_i depletion promoted the expression of P-transporter (Figure 2A) and phosphatase genes (Figure 2C). The positive relationship between the transcripts of *phoD* and *pstS* in the cultures ($r = 0.7$, $P < 0.01$, $n = 16$) indicated that increases in P_i transport and the catalysis of DOP are synchronous. In fact, the expression levels of the genes involved in P uptake and transport were upregulated when P was deficient. After P starvation and prolonged P_i limitation, 246 and 823 genes were identified to be differentially expressed in *Anabaena* sp. strain 90, respectively, suggesting that pho regulon genes are as important as the APase gene commonly used for monitoring P_i status.[45]

The expressions of *pstS* and *phoD* were sensitive to P supply. Relative to +P treatment, *pstS* was significantly upregulated

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during −P treatment. After N addition, this expression was further upregulated (Figure 2A). The expression of *phoD* was significantly upregulated by the −P treatment (Figure 2C). The induction of APase corresponded with *phoD* expression. Total APA was negatively and significantly related to SRP concentration ($r = -0.8$, $P < 0.01$, $n = 16$). We were not able to exclude the contribution of bacteria in the cultures to the total APA, while the clearly higher percentage of ELF-labeled cells in −P cultures than in +P cultures reflected APA regulation by the environmental P supply. When ambient P_i concentrations declined, cultures of both *Nodularia* and *Aphanizomenon* displayed higher APA.[46] In Lake Kinneret, P bioavailability strongly affected the growth of *Aphanizomenon ovalisporum*. The uptake of P_i was faster under P_i-depleted conditions than in P_i-sufficient cells.[47] Similarly, *D. flos-aquae* had a high affinity for P_i and rapidly hydrolyzed DOP. Additionally, N played an important role during P uptake. Expression of *pstS* and *phoD* was significantly upregulated after N addition in −P cultures. In a field study, the N_2-fixing cyanobacterium *Cylindrospermopsis raciborskii* yielded a higher biomass under P limitation conditions where a sufficient supply of NO_3^--N was maintained.[48] Limitation of N in *Dolichospermum* may reduce the uptake of P.[49] Considering that the P-transporter and APase are both N-rich proteins, N addition may enhance their expression and thus support the ability to overcome P limitation.

Adaptation to P limitation also includes the ability to take up P_i in excess of its immediate cellular requirements under P_i-sufficient conditions and to store it in the form of PPBs.[31] Unlike formerly described mechanisms of forming PPBs, which accumulate in P-replete conditions and sustain cell growth when cells suffer from P depletion, *D. flos-aquae* did not form PPBs in +P or −P treatments under N_2-fixation conditions. However, N addition caused an abrupt rise in the amount and percentage of PPB-containing cells in the +P treatment, suggesting that PPB formation was related to N supply. Consistent with our observation, when nitrate was supplied as the N source, poly-P levels increased from 3% to 15% of total cell P concentration, indicating that inorganic N in the ambient water controlled the accumulation of poly-P in *D. flos-aquae*.[38] Since poly-P synthesis requires energy,[30] N_2 fixation in cyanobacteria may compete with reserve P accumulation for ATP. N addition may relieve the competition and greatly enhance poly-P formation under P-replete conditions. In summary, N plays a vital role in the P-scavenging strategy, and N bioavailability may help N_2-fixing cyanobacteria overcome P limitation. With regard to N loading of natural waters, it is still important for N_2-fixing cyanobacteria although they fix N_2. Actually, N_2-fixing cyanobacteria may also suffer from N limitation. Flecker et al.[49] observed that the N_2-fixing cyanobacteria genus *Anabaena* was one of the taxa that responded most dramatically to the addition of N in an Andean stream.[49] Furthermore, a long-term study on whole-ecosystem nutrient enrichment indicated that the amount of N fixed by cyanobacteria was not adequate to offset the reduction of exogenous N inputs to the waterbody and that the dynamics of both N and P are significant in controlling harmful algal blooms.[6,8] On the other hand, N_2-fixing might be inhibited by P deficiency. Additionally, N_2 fixation is controlled by other factors such as light, trace metals, organic matter availability, and turbulence in the surface waters of lakes having cyanobacterial blooms.[50] To avoid light limitation and the interactive effects between P availability and irradiance, we chose a suitable irradiance of 30 μmol photons m^{-2} s^{-1} for *D. flos-aquae* according to the suggestion from the FACHB-collection and our previous experience with this genus.[51] In light of our results, we predict that the paradigm of N loading reduction stimulating N_2-fixing cyanobacterial blooms occurs only when P loading is high.

P may influence the growth of N_2-fixing cyanobacteria through the energy supply during heterocyst formation, while N availability could help them overcome P limitation by synthesizing a P-transporter and an extracellular phosphatase, which suggests the mutual dependence of N and P metabolisms. Our results emphasize the importance of dual N and P reduction in controlling cyanobacterial blooms on the molecular and biochemical scales.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b04992.

Supplemental methods for DNA extraction, cloning, sequencing, and phylogenetic analyses. Figure S1. Phylogenetic analysis of *Dolichospermum flos-aquae* and model cyanobacteria based on partial 16S rRNA sequences. Figure S2. Phylogenetic analysis of *Dolichospermum flos-aquae* and model cyanobacteria based on partial *phoD* gene sequences. Figure S3. Phylogenetic analysis of *Dolichospermum flos-aquae* and model cyanobacteria based on partial *pstS* gene sequences (PDF)

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