Cyanophycin accumulated under nitrogen-fluctuating and high-nitrogen conditions facilitates the persistent dominance and blooms of Raphidiopsis raciborskii in tropical waters

Zhe Lu [a], Jinmei Ye [a], Zhijiang Chen [a], Lijuan Xiao [a], Lamei Lei [a,*], Bo-ping Han [a], Hans W. Paerl [b]

[a] *Department of Ecology and Institute of Hydrobiology, Jinan University, Guangzhou, China*
[b] *Institute of Marine Sciences, The University of North Carolina at Chapel Hill, Morehead City, United States of America*

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ABSTRACT

Nutrient storage is considered a critical strategy for algal species to adapt to a fluctuating nutrient supply. Luxury phosphorus (P) uptake into storage of polyphosphate extends the duration of cyanobacterial dominance and their blooms under P deficiency. However, it is unclear whether nitrogen (N) storage in the form of cyanophycin supports persistent cyanobacterial dominance or blooms in the tropics where N deficiency commonly occurs in summer. In this study, we examined genes for cyanophycin synthesis and degradation in *Raphidiopsis raciborskii*, a widespread and dominant cyanobacterium in tropical waters; and detected the cyanophycin accumulation under fluctuating N concentrations and its ecological role in the population dynamics of the species. The genes for cyanophycin synthesis (*cphA*) and degradation (*cphB*) were highly conserved in 21 out of 23 *Raphidiopsis* strains. This suggested that the synthesis and degradation of cyanophycin are evolutionarily conserved to support the proliferation of *R. raciborskii* in N-fluctuating and/or deficient conditions. Isotope ^{15}N-NaNO$_3$ labeling experiments showed that *R. raciborskii* QDH7 always commenced to synthesize and accumulate cyanophycin under fluctuating N conditions, regardless of whether exogenous N was deficient. When the NO_3^--N concentration exceeded 1.2 mg L^{-1}, *R. raciborskii* synthesized cyanophycin primarily through uptake of ^{15}N-NaNO$_3$. However, when the NO_3^--N concentration was below 1.0 mg L^{-1}, cyanophycin-based N was derived from unlabeled N_2, as evidenced by increased dinitrogenase activity. Cells grown under NO_3^--$N < 1.0$ mg L^{-1} had lower cyanophycin accumulation rates than cells grown under NO_3^--$N > 1.2$ mg L^{-1}. Our field investigation in a large tropical reservoir underscored the association between cyanophycin content and the population dynamics of *R. raciborskii*. The cyanophycin content was high in N-sufficient (NO_3^--$N > 0.45$ mg L^{-1}) periods, and decreased in N-deficient summer. In summer, *R. raciborskii* sustained a relatively high biomass and produced few heterocysts ($< 1\%$). These findings indicated that cyanophycin-released N, rather than fixed N, supported persistent *R. raciborskii* blooms in N-deficient seasons. Our study suggests that the highly adaptive strategy in a N_2-fixing cyanobacterial species makes mitigating its bloom more difficult than previously assumed.

1. Introduction

Cyanobacterial blooms are occurring with increased frequency, intensity, and duration in freshwater bodies throughout the world. These blooms threaten human health and freshwater ecosystem functioning (Coffer et al., 2021; Ho et al., 2019; Huisman et al., 2018; Paerl and Otten, 2013). Many species of cyanobacteria have evolved various eco-physiological strategies to adapt to nutrient fluctuations. These strategies allow them to dominate the phytoplankton community and form blooms (Paerl and Otten, 2013; Scott et al., 2019). One of the well-studied strategies is luxury P uptake into storage as polyphosphate (polyP), facilitating the dominance and persistence of cyanobacteria in P-deficient environments (Carey et al., 2012; Ritchie et al., 2001). Another physiological strategy is N_2 fixation *via* conversion of N_2 to NH_4^+, which can be used as a N source when available N sources are depleted in waters (Schindler et al., 2008). However, N_2 fixation is energetically expensive (Dixon and Kahn, 2004). A growing body of evidence indicates that N_2 fixation is not the principal strategy for acquiring N, and unlikely provides enough N to support cyanobacterial blooms (Paerl et al., 2016; Scott et al., 2019; Shatwell and Köhler, 2019;

* Corresponding author.
E-mail address: tleilam@jnu.edu.cn (L. Lei).

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Xu et al., 2021). Therefore, there are likely other mechanisms allowing cyanobacteria to gain a competitive advantage under N-deficient and/or fluctuating N conditions.

Several earlier laboratory studies showed that cyanobacteria can store N, just as they store P (Allen et al., 1980; Simon, 1971). Recently, it was found that the N reserves are mainly in the forms of cyanophycin and phycobilisomes in many cyanobacterial species, and cyanophycin is used as an internal N source under N-deficient conditions before using phycobilisomes (Zhang and Yang, 2019). Phycobilisomes plays a less important role than cyanophycin in the diazotrophic cyanobacteria which use cyanophycin as the main N storage (Li et al., 2001). Cyanophycin ($C_{10}H_{19}N_5O_5$) is a non-ribosomally synthesized polypeptide with a relatively high ratio of N to C (Flores et al., 2019). Its biosynthesis and degradation are catalyzed by cyanophycin synthase (encoded by *cphA*) and cyanophycinase (encoded by *cphB*), respectively (Picossi et al., 2004). Under sufficient N supply, many cyanobacteria accumulate cyanophycin during unbalanced growth induced by nutrient fluctuations, e.g., P pulses (Polerecky et al., 2021; Trautmann et al., 2016; Van de Waal et al., 2010). In contrast, in the absence of N, cyanobacteria contain very low levels of cyanophycin (Mackerras et al., 1990). Cyanophycin accumulation enables *Synechocystis* sp. and *Microcoleus* sp. to optimize N assimilation under N-deficient conditions, and especially under fluctuating N supply (Tee et al., 2020; Watzer and Forchhammer, 2018). Cyanophycin has been detected in many cyanobacteria species but never in eukaryotic algae (Mojzes et al., 2020). Therefore, analogous to the case of P storage, cyanophycin may provide cyanobacteria with a competitive advantage over sympatric phytoplankton species in nature with fluctuating or deficient N supply (Forchhammer and Schwarz, 2019).

Several bloom-forming cyanobacteria such as *Microcystis* sp., *Dolichospermum* sp. (formerly *Anabaena*), *Aphanizomenon ovalisporum*, and *Planktothrix agardhii* store N intracellularly as cyanophycin (Sukenik et al., 2015; Van de Waal et al., 2010). Their advantage over eukaryotic algae under N-limiting conditions may be related to cyanophycin (Kurmayer et al., 2016). Freshwater diazotrophic, filamentous *Raphidiopsis* (formerly *Cylindrospermopsis*) *raciborskii* is one of the most successful bloom-forming cyanobacteria in the tropics (Lei et al., 2014; Lu et al., 2021; Rzymski and Poniedzialek, 2014). This species has diverse nutritional utilization strategies that are critical for its rapid growth and global expansion (Burford et al., 2016). *R. raciborskii* was reported to have a competitive advantage under low-N conditions (Figueredo et al., 2014; Xiao et al., 2021). As N_2 fixation by *R. raciborskii* accounts for less than 10% of the total assimilated N, and it cannot support a high growth rate under N deficiency (Willis et al., 2016). In addition to fixed N, there must be other N sources to meet the high N demands of dominant or blooming *R. raciborskii* under N-deficient conditions.

Previous studies have shed some light on the N storage in *R. raciborskii* (Mackerras et al., 1990; Trautmann et al., 2016; Van de Waal et al., 2010); however, there are several major issues still to be resolved. First, the heterocysts of *R. raciborskii* CS-505 can accumulate cyanophycin polar "nodules" from fixed N in N-free medium (Plominsky et al., 2015), but it is unknown whether the ability to synthesize this N storage compound is restricted to strain CS-505 or common among *R. raciborskii* taxa. There are multiple ecotypes with a high trait variability within and among locally occurring *R. raciborskii* strains (Bolius et al., 2017). Second, cyanophycin is considered to also be accumulated during the N_2 fixation process in diazotrophic cyanobacteria (Watzer and Forchhammer, 2018), but it is unclear whether *R. raciborskii* can accumulate cyanophycin under a fluctuating N supply (namely, N-sufficient and N-deficient conditions), as has been reported for non-diazotrophic cyanobacteria (Flores et al., 2019). Third, observations on cyanophycin synthesis in cyanobacteria were predominantly conducted under standard laboratory conditions (Burnat et al., 2014; Sukenik et al., 2015; Van de Waal et al., 2010). Thus, probing into the relationship between N concentration and cyanophycin accumulation in natural conditions will provide further insights into the functional significance of cyanophycin in phytoplankton population dynamics.

To investigate the N stored in cyanophycin for *R. raciborskii* to respond to N-fluctuating, we first checked whether all the published full genomes of *R. raciborskii* strains contain genes involved in cyanophycin synthesis and degradation (*cphA* and *cphB*, respectively). Subsequently, we conducted culture experiments to examine cyanophycin accumulation and dinitrogenase activity in *R. raciborskii* under two N-fluctuation scenarios. We also quantitatively detected the dynamics of cellular cyanophycin content, nutrient levels and phytoplankton biomass in a large reservoir in South China, which has a stable annual phytoplankton community dominated by *R. raciborskii* (Xiao et al., 2021). We hypothesized that *R. raciborskii* assimilates ambient N and stores it as cyanophycin during N sufficient period, and that it is this stored N, rather than energy-intensive fixed N, that supports proliferation of *R. raciborskii* in N-deficient waters.

2. Materials and methods

2.1. Verification of cyanophycin associated genes and granules in *Raphidiopsis*

First, we compared the genomic sequences of 23 *Raphidiopsis* strains to verify the presence of *cphA* and *cphB*. These genomic sequences were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/genome). The *Raphidiopsis* strains were isolated from diverse ecoregions around the world (Table 1). Then, we conducted metabolic-level analysis of *Raphidiopsis* cells containing cyanophycin granules. We extracted cyanophycin as described by Trautmann et al. (2016) with some modifications (Fig. S1A).

2.2. Exploring the cyanophycin accumulation in two simulated N-fluctuation scenarios with stable isotope ^{15}N-$NaNO_3$

2.2.1. Strain and culture medium

Two simulated N-fluctuation scenarios in which sufficient N concentration changed to N-deficient conditions were applied to a 30-day culture of *R. raciborskii* QDH7, a strain isolated from Qiandeng Lake (23°03′ N; 113°08′ E), Foshan City, Guangdong province, China, and was preserved at Jinan University. This strain was unialgal but not axenic. During experimental work, the absence of heterotrophic bacteria was confirmed by routine microscopic observation. We chose the form

Table 1
The verification of genes *cphA* and *cphB* encoding cyanophycin synthetase and cyanophycinase in 23 *Raphidiopsis* strains.

Strains	Sources	*cphA*	*cphB*
C03	Australia	+++	+
C04		+++	+
C07		+++	+
CS-505		+++	+
CS-506		+++	+
CS-508		+++	+
S01		+++	+
S05		+++	+
S06		+++	+
S07		+++	+
S10		+++	+
Cr2010	USA	+++	+
KL1		+++	+
GIHE2018	Korea	+++	+
LB2897	Uruguay	+++	+
MVCC14		+++	+
MVCC19		+++	+
CENA302	Brazil	+++	+
CENA303		++	+
CYRF		n.d.	n.d.
CYLP		n.d.	n.d.
N8	China	+++	+
QDH7		+++	+

and concentration of dissolved N based on observations in the reservoir where we conducted our field study. However, because the simulated N fluctuation scenario was achieved by dilution with N-free medium on day 13, if the initial N concentration was 1.4 mg L^{-1}, then the exogenous N concentration on day 13 (< 0.3 mg N L^{-1}, Fig. S2) was too low. Therefore, it was finally decided to supply nitrate at an initial N concentration of 14 mg L^{-1}. The basal medium was modified Wright's cryptophyte (WC) medium, which contained 1000 mL distilled water, 36.76 mg $CaCl_2 \bullet 2H_2O$, 36.97 mg $MgSO_4 \bullet 7H_2O$, 12.60 mg $NaHCO_3$, 0.15 mg K_2HPO_4, 85.01 mg $NaNO_3$, 28.42 mg $Na_2SiO_3 \bullet 9H_2O$ and trace metals (Guilard et al., 1974).

2.2.2. Experimental design

R. raciborskii QDH7 were first cultivated in N-free WC medium for 2 days to exhaust the initial N in cells (Qian et al., 2017) till no growth was observed. This eliminated any effect of simultaneous uptake of various DIN sources. The conventional $NaNO_3$ in the modified WC medium was replaced with ^{15}N-$NaNO_3$ as the sole N source for isotopic experiments. The N-starved cells were inoculated into the above modified WC medium (^{15}N = 14 mg L^{-1}) with an initial optical density of 0.1 – 0.12 at 750 nm ($OD_{750\ nm}$). Then, on day 13, the N concentration in the culture medium was reduced by four times by adding N-free WC medium (one-off dilution); or by two times on day 13, then two times on day 15, and then two times on day 18 (stepwise dilution). Cells cultured in N-free WC medium served as the control. The ambient nitrate concentration was determined by measuring $OD_{220\ nm}$ and calculated using the following equation: c (NO_3^-) (mM) = 0.2726 × $OD_{220\ nm}$ − 0.0024 (R^2 > 0.99) (Collos et al., 1999). The culture experiments were performed in tissue culture flasks of 250 mL, with 60 mL culture medium (Fig. S3) at 25 ± 1 °C under 35 ± 5 μmol photons m^{-2} s^{-1} with a 12 h:12 h light: dark cycle. Each treatment was performed in triplicate. For each replicate sample, the culture in each flask was only used to determine cell growth (1 mL), and cyanophycin content (50 mL) or dinitrogenase activety (40 mL) on a sampling day (Fig. 1A).

2.2.3. Determination of cell growth, cyanophycin content, and dinitrogenase activity

Cell growth was determined by an estimation from $OD_{750\ nm}$ every 3 days using a spectrophotometer (UH5300, Hitachi, Tokyo, Japan) (Briand et al., 2008). There is a good linear relationship between $OD_{750\ nm}$ and dry biomass/cell concentration (Post et al., 1985; Fig. S4). At 3-day intervals, the cyanophycin content in 50 mL culture samples was measured. The culture samples were immediately filtered through an acetate membrane (0.45 μm pore size), and then the membrane containing the cells was stored at −80 °C until cyanophycin content (% of *R. raciborskii* dry cell weight) analysis. Algal samples of 40 mL were withdrawn aseptically on day 6, 12, 15, and 21 for detection of dinitrogenase activity, which was detected by acetylene reduction assay (Paerl et al., 2014). Briefly, culture samples were injected into crimp-sealed 100 mL serum bottles previously flushed with N_2. CaC_2-generated acetylene (10%, *v*/*v*) of 7 mL was injected into these sample bottles. The sample bottles were incubated in a constant temperature water bath at 30 °C for 4 h. Subsequently, the gas phase samples were collected in pre-evacuated Vacutainer (Becton Dickinson Inc.) with a gas-tight syringe (Hamilton Co., Reno, USA). The product, ethylene content was determined using a Shimadzu flame ionization gas chromatograph (model GC-14A) equipped with a GS-Q capillary column (Agilent J & W Scientific, Folsom, CA; 30 *m* × 0.546 mm). One unit (U) of dinitrogenase activity was defined as the amount of dinitrogenase catalyzing the reduction of 1 nmol of acetylene per hour per mg fresh biomass.

2.3. Examining the effect of ^{15}N-$NaNO_3$ concentrations on source of N used in cyanophycin synthesis

In diazotrophic cyanobacteria, the N used in cyanophycin synthesis

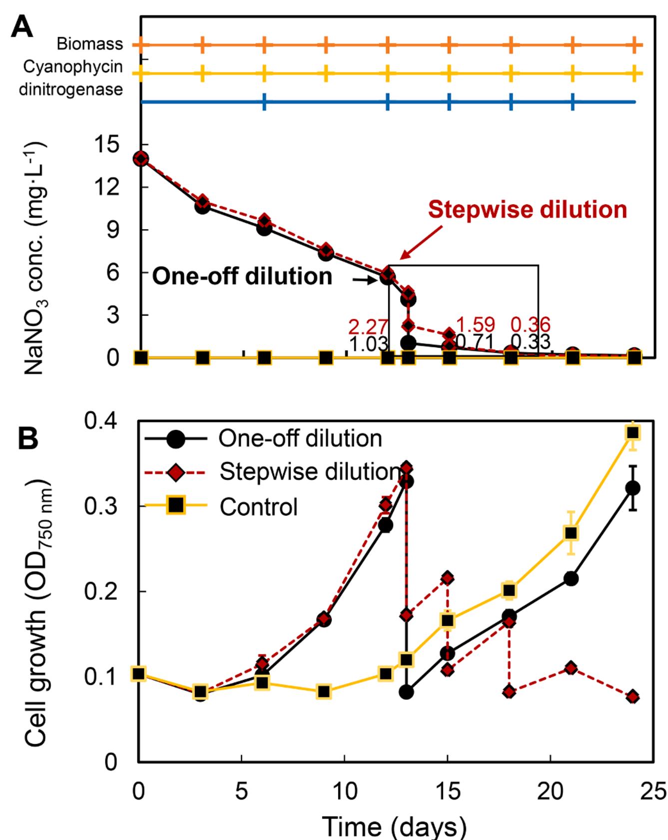

Fig. 1. Changes in exogenous NaNO3concentration and cell growth of *R. raciborskii*QDH7 over time under two simulated fluctuating N conditions. (A) Exogenous NaNO3concentration, and the parameters and their sampling days. (B) Cell growth, indicated by OD750 nm. The yellow curve represents the N-deplete condition, namely the control group. The black and red ones mean the treatments of one-time dilution and stepwise dilution, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

can be derived not only from ambient N (Van de Waal et al., 2010), but also from newly fixed N (Finzi-Hart et al., 2009). To explore the effect of N concentrations on the source of N used in cyanophycin synthesis, we supplied ^{15}N-$NaNO_3$ at concentrations of 0.8, 1.0, 1.2, and 1.4 mg N L^{-1} in *R. raciborskii* QDH7 culture medium. The N-starved cells were prepared as described above and inoculated into these ^{15}N-modified WC media with an initial $OD_{750\ nm}$ of 0.1. The cells were sampled on day 0, 1, 3, 10, and 20 for cyanophycin extraction and detection as described above. Because cyanophycin is a polymer of arginine and aspartate, the ^{15}N-arginine content can indicate whether the N in cyanophycin derives from NO_3^-–^{15}N or ^{14}N derived from N_2 fixation. Samples were collected on day 1 and day 3 for ^{15}N-arginine detection. The concentration of ^{15}N-arginine in the solution (containing the solubilized cyanophycin) was determined by Daruibiotech Co. Ltd. (Guangzhou, China). The parent ions of the normal molecule (*m/z* = 175) and those of ^{15}N-labelled arginine (*m/z* = 176 (containing one atom of ^{15}N), *m/z* = 177(two atoms of ^{15}N), *m/z* = 178 (three atoms of ^{15}N), $[M+H]^+$) were detected.

2.4. Field surveys

2.4.1. Study reservoir and sampling

To explore the relationship between cyanophycin accumulation and *R. raciborskii* population dynamics in natural conditions, a monthly limnological investigation of nutrient concentrations, phytoplankton composition, proportion of heterocysts (% of total *R. raciborskii* cells),

and cyanophycin content (% of *R. raciborskii* dry cell weight) in the water was conducted from January 2019 to December 2019 in a tropical reservoir, the ZH Reservoir (22°57′ N, 112°57′ E, Fig. 4A) in South China. Due to the monsoonal climate, the reservoir has a warm monomictic mixing regime. Its normal storage volume is about 1.2◊10^8 m^3, its maximum depth is 12 m, and it has dendritic shape. The sampling site was in the center of the reservoir (Fig. 4A). At this site, water samples were collected at a depth of 0.5 m and kept cool until analyses.

2.4.2. Nutrient levels in water, phytoplankton composition, and cyanophycin analysis

For nutrient analysis, water samples were transported to the laboratory within 8 h of collection. The environmental nutrients included soluble reactive P (SRP), nitrate (NO_3^- -N), ammonium (NH_4^+-N), TN and total P (TP). DIN was calculated as the sum of NO_3^- -N and NH_4^+-N. The nutrient concentrations were measured and calculated following the standards set by the USA Environmental Protection Agency. For phytoplankton composition and biomass analysis, each 1000 mL water sample was preserved in the field with 1% *v/v* Lugol's iodine solution and sedimented for 48 h. Phytoplankton taxa were identified according to the latest taxonomic literature, and counted under an inverted microscope (at 400 × magnification) (Utermöhl, 1958). At least 400 settling units were counted, and the average sizes of all the counted algal cells were calculated. The biomass of each algal cell was calculated based on biovolume, assuming specific gravity of 1 mg mm^{-3} (Hillebrand et al., 1999). For cyanophycin content analysis, each 200 mL water sample was filtered through an acetate membrane (0.45 μm pore size). Cyanophycin was extracted from the algae collected on the membranes. The cyanophycin content (% of *R. raciborskii* dry cell weight) was calculated using the following formula:

$$CPGc(\%dcw) = \frac{C_{CPG}(\mu g{\cdot}mL^{-1}) \times V_{CPG}(mL)}{B_{rap}(mg{\cdot}L^{-1}) \times V_w(mL) \times 10\% \times 1000} \times 100\%$$

where CPGc represents the cyanophycin content (% of *R. raciborskii* dry cell weight); C_{CPG} (μg•mL^{-1}) is the cyanophycin concentration in the extract solution; V_{CPG} (mL) is volume of the extract solution; B_{rap} (mg•L^{-1}) is the biomass concentration of *Raphidiopsis* sp. in the ZH Reservoir; V_w (mL) is the volume of water samples; and 10% is an empirical value representing the proportion of dry cell weight out of total fresh algal biomass (Reynolds, 2006).

2.5. Statistical analyses

Data shown in all the figures and tables are average values and standard deviations of three biological replicates. A repeated measures analysis of variance (ANOVA) was applied to test differences among dilution treatments and time and their interaction for cell growth, $NaNO_3$ concentration (before dilution on day 13) in the Experiment 2.2; for cyanophycin content, dinitrogenase activity, and ^{15}N-arginine content (μmol•L^{-1}) in the extracted cyanophycin in the Experiments 2.2 and 2.3. A Tukey's post hoc multiple comparison was used to examine the between-group (one-off, stepwise dilution and control group) differences at the individual timepoints and within-group changes over time in cell growth, cyanophycin, dinitrogenase activity, and ^{15}N-arginine content (μmol•L^{-1}) in the extracted cyanophycin using the multcomp package in R. A paired *t* test was performed to analyze the significantly statistical differences in the N concentration and cyanophycin contents (% of dry cell weight) between the first half year and the second half year.

3. Results

3.1. Cyanophycin is prevalent in Raphidiopsis

We confirmed the presence of three putative cyanophycin synthase genes (*cphA, cphA2* and *cphA2'*) and one putative cyanophycinase gene (*cphB*) in the draft genomes of *R. raciborskii* QDH7, N8 and publicly available genomic data for 21 *R. raciborskii* strains isolated around the world. The genes *cphA* and *cphB* were widely present among strains of *R. raciborskii*, including strains from China, Australia, Korea, and the USA, but not in the strains CYRF and CYLP from Brazil (Table 1). The lack of *cphA* and *cphB* in the genomes of the two Brazilian strains CYRF and CYLP might be because of incomplete genome assembly and annotation. Both *cphA* and *cphB* were highly conserved among *R. raciborskii* strains. The three putative *cphA* genes were around 2654, 989, and 1940 bp in length, and *cphB* was around 893 bp in length (Table S1). Cyanophycin granules were observed inside *R. raciborskii* cells (Fig. S1B). These results confirmed that *R. raciborskii* accumulates cyanophycin as a form of stored N. We speculated that cyanophycin is evolutionarily conserved to support the proliferation of *R. raciborskii* in N-fluctuating and/or deficient conditions. To test this idea, we conducted further experiments and observations to determine the contribution of N stored as cyanophycin to the persistent dominance and blooms of harmful cyanobacteria.

3.2. Growth and N assimilation patterns under simulated N-fluctuating conditions

The growth patterns of *R. raciborskii* in two simulated N-fluctuation scenarios were shown in Fig. 1B and S5. The biomass (indicated by $OD_{750\ nm}$) was not significantly different between these two N-dilution scenarios except for day 12 (repeated measures ANOVA: $p = 0.963$, 0.996, 0.991, 0.159, on day 3, 6, 9, 13, respectively, Table S2, S3) before dilution (namely, before day 13). The biomass was significantly higher under N-fluctuating conditions than under N-depleted conditions at each timepoint (from day 9, $p < 0.001$, repeated measures ANOVA). Without N supplementation, there was a long lag in cell growth (Fig. 1B), and the biomass did not increase until day 13. The N-uptake curves before dilution were similar in these two N-dilution scenarios, as indicated by their $NaNO_3$ concentration (repeated measures ANOVA, $F = 4.001$, $p = 0.056$, Fig. 1A and Table S2). In the first 3 days, exogenous N was rapidly taken up by *R. raciborskii* QDH7, and the uptake rate of $NaNO_3$ was highest at this time (Fig. S6). Cyanophycin content on day 3 was significantly higher than day 0 (Tukey's post hoc: $p < 0.001$, in one-off dilution, stepwise group, and control group, respectively) when the N-starved cells were inoculated into fresh N-sufficient medium (Fig. 2A). The cyanophycin content of *R. raciborskii* QDH7 increased to 20.8% (of dry cell weight). After that, the cyanophycin content gradually decreased until day 13, when the cell culture medium was diluted. Upon N fluctuation caused by dilution, cyanophycin was accumulated again (Fig. 2A). After this one-off dilution, the cyanophycin content was significantly higher on day 15 than on day 12 (Tukey's post hoc: $p < 0.01$). Similarly, upon stepwise dilution, the cyanophycin content increased from day 15 to day 18, and from day 18 to day 21. Day 18 and day 21 were the days when the stepwise dilutions occurred. Notably, under N-depleted conditions, cyanophycin was unexpectedly accumulated on day 3, and its level was not significantly different from that under N-sufficient conditions (Tukey's post hoc: $p = 0.183$, compared to one-off dilution; $p = 0.863$, compared to stepwise dilution, Fig. 2A). However, from day 3 onwards, without N dilution, the cyanophycin content decreased with increasing culture time (Fig. 2A). The activity of dinitrogenase was highly dependent on the exogenous NO_3^- concentration (Fig. 2B). For instance, its activity was remarkably higher in the early stage of N-depleted (blank control) conditions. Since the N concentration dropped to 1.03 mg L^{-1} after one-off dilution on day 13 (Fig. 1A), the dinitrogenase activity on day 15 significantly increased (Tukey's post hoc: $p < 0.001$, Fig. 2B) compared with that in the stepwise-dilution group, in which the NO_3^--N concentration was 2.27 mg L^{-1} on day 13 (Fig. 1A). After the N concentration dropped to 0.33 mg L^{-1} due to stepwise dilution on day 18, a significant increase in dinitrogenase activity (Tukey's post hoc: $p < 0.001$, compared to one-

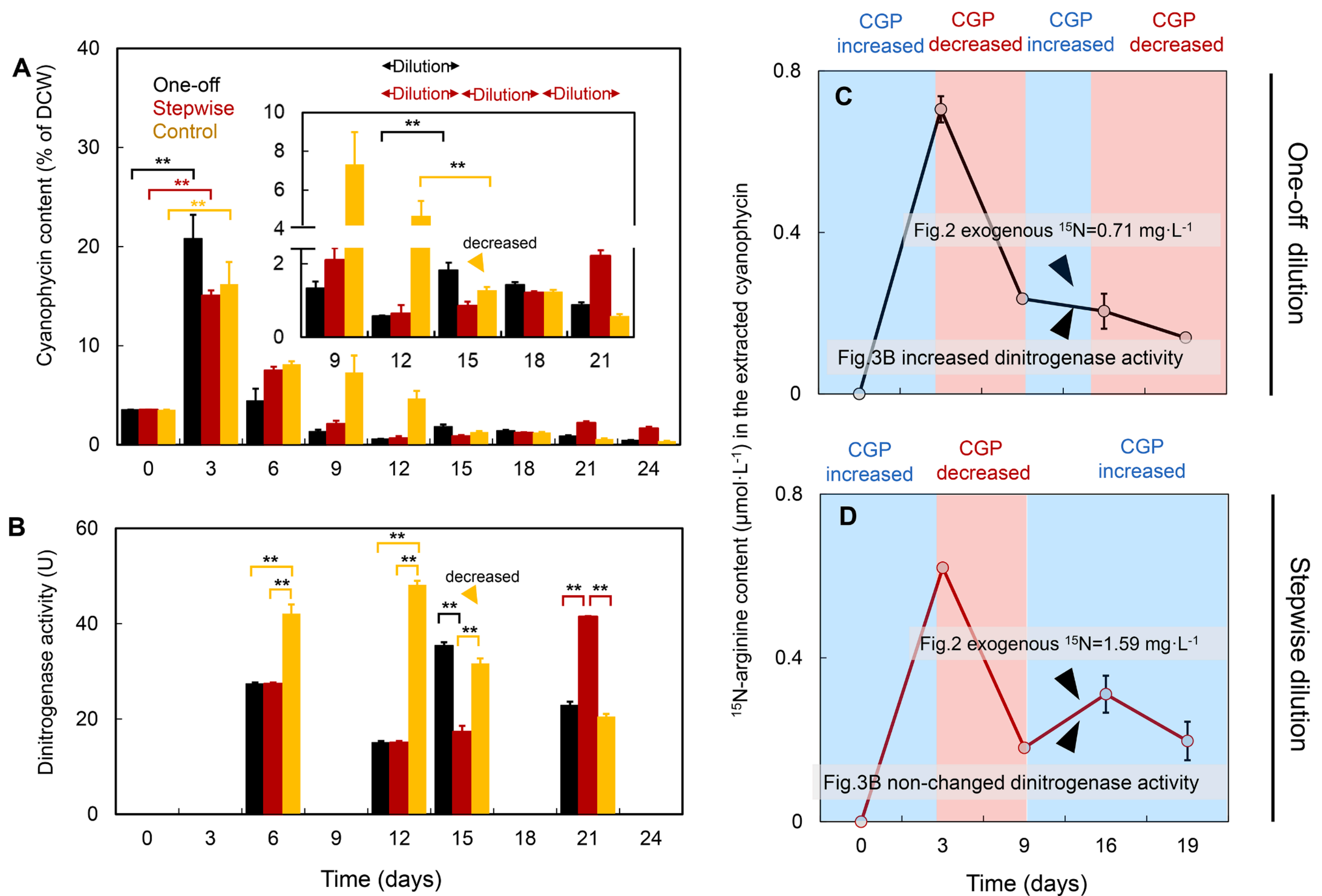

Fig. 2. N assimilation patterns of *R. raciborskii*under two simulated fluctuating conditions. (A) The cyanophycin content (% of dry cell weight). (B) The dinitrogenaseactivities (U). (C–D) The 15 N arginine content (μmol•L-1) in cyanophycin; C–One-off dilution, D–Stepwise dilution. The color of background represents the variation tendency of cyanophycin (CGP). Blue, cyanophycin contents increased; Red, decreased. Values were represented as mean ±s.d.($n = 3$). **, Significance at 0.01 level (Tukey's post hoc multiple comparison).

off dilution and control group, respectively) was detected on day 21.

3.3. The source of N used for cyanophycin synthesis under fluctuating N at different concentrations

The source of N used in cyanophycin synthesis depended on the concentration of exogenous bioavailable NO_3^--N. As above, there was non-significant difference in exogenous NO_3^--N concentration between the two N-fluctuating conditions before day 13 (repeated measures ANOVA, $F = 4.001$, $p = 0.056$, Fig. 1A and Table S2). Accordingly, there was no significant difference in the ^{15}N-arginine content of cyanophycin between the two treatments (Tukey's post hoc: $p = 0.797$, on day 3; $p = 0.915$, on day 9, Fig. 2C, D). In the one-off dilution group, the exogenous NO_3^--N concentration decreased to 0.71 mg L^{-1} on day 15, accompanied by the accumulation of cyanophycin and the up-regulation of dinitrogenase activity (Fig. 2A, B); however, the content of cyanophycin-based ^{15}N-arginine dropped on day 16 (Fig. 2C). In contrast, the exogenous NO_3^--N concentration did not decrease notably in the stepwise dilution group on day 15 (concentration, 1.59 mg L^{-1}). At this NO_3^--N concentration, dinitrogenase activity was not highly upregulated on day 15 (Fig. 2B), but the cyanophycin-based ^{15}N-arginine content (Fig. 2D) and cyanophycin content synchronously increased (Fig. 2A). The experiments with different ^{15}N-$NaNO_3$ concentrations revealed a specific N threshold that determined the source of cyanophycin-based N (Fig. 3). When cells were cultured with 1.2 and 1.4 mg NO_3^--N•L^{-1}, the cyanophycin was rapidly accumulated at first and then gradually decreased. In contrast, the cyanophycin content in cells cultured with 0.8 and 1.0 mg NO_3^--N•L^{-1} did not increase rapidly on day 1 (Tukey's post hoc: $p < 0.05$, compared to that on day 0), but increased on day 3 (Tukey's post hoc: $p < 0.05$, compared to that on day 1) (Fig. 3A). Tracing of ^{15}N-arginine revealed, significantly higher content in the cyanophycin extracted from cells in the 1.2 and 1.4 mg NO_3^--N•L^{-1} groups than in the other groups (Fig. 3B), indicating that ambient ^{15}N-$NaNO_3$ was the main source of N in the newly synthesized cyanophycin. In contrast, the ^{15}N-arginine content in cyanophycin was significantly lower on day 1 (Tukey's post hoc: $p < 0.01$) and day 3 (Tukey's post hoc: $p < 0.05$) in the 0.8 and 1.0 mg N L^{-1} groups compared to 1.4 and 1.2 mg NO_3^--N•L^{-1} groups.

3.4. Nutrient, phytoplankton, cyanophycin and heterocyst profiles in the zh reservoir

The TN concentration fluctuated in the reservoir throughout 2019, but its average in the first half of the year (1.34 mg N L^{-1}) was significantly higher than that in the second half (1.03 mg N L^{-1}) (paired *t*-test, $t = 2.79$, df $= 8.90$, $p = 0.02$, Fig. 4C). In June, the TN concentration decreased precipitously to 0.89 mg L^{-1}. Similarly, the NO_3^--N concentration remained relatively stable in the first half of the year (average approx. 0.45 mg N L^{-1}), and then dropped to the annual minimum of $<$ 0.05 mg N L^{-1} in June (Fig. 4C) before the onset of *R. raciborskii* blooms (Fig. 4D). The NH_4^+ concentration remained stable at around 0.06 mg L^{-1} despite a slight decrease early on (Fig. 4C). As for phytoplankton

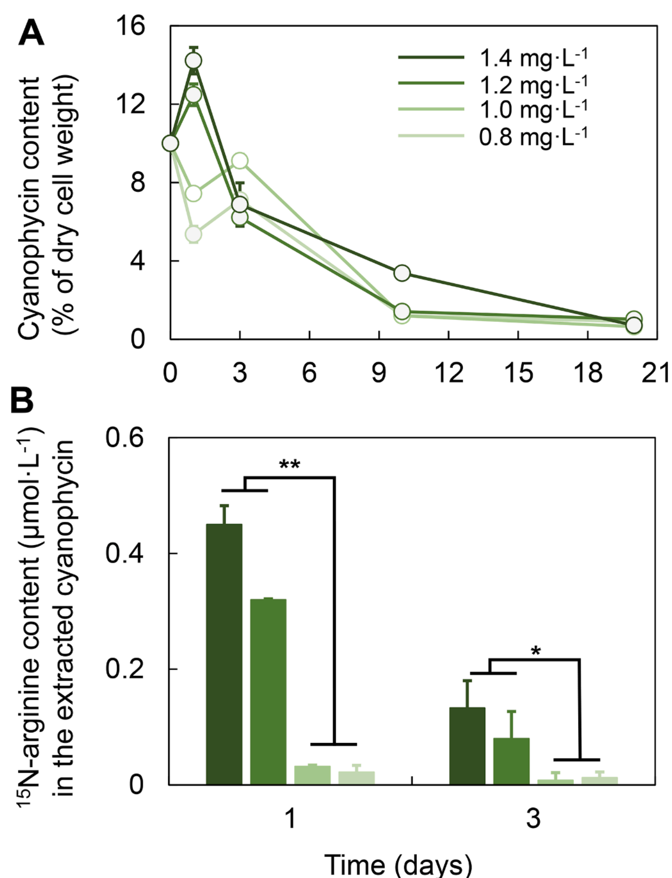

Fig. 3. Changes of the cyanophycin contents and its 15N-arginine contents in *R. raciborskii*QDH7 under different 15N-NaNO3concentrations. (A) The cyanophycin content (% of dry cell weight). (B) The 15 N arginine content (μmol•L-1) in the extracted cyanophycin. The gradient of green colors from dark to light green represents the concentration gradient of NaNO3, from 1.4 mg L-1to 0.8 mg L-1. * and **, Significance at 0.05 and 0.01 level, respectively (Tukey's post hoc multiple comparison). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

biomass, *R. raciborskii* was dominant in the phytoplankton community throughout the entire year, accounting for nearly 80% of the phytoplankton biomass (Fig. 4D). The phytoplankton biomass (including that of *R. raciborskii*) peaked in July after the TN concentration peaked in May. Starting from May, the phytoplankton biomass (including that of *R. raciborskii*) remained higher than that in spring (from January to April). Interestingly, the cyanophycin dynamics were diametrically opposite to *R. raciborskii* population dynamics (Fig. 4B). The cyanophycin content (% dry cell weight) was dependent on the ambient N levels, so it was relatively high in the first half of the year (e.g., peak content of 5.0% in February). The cyanophycin content decreased after May, and its average content was significantly decreased in the second half of the year (paired *t*-test, $t = 3.242$, df $= 5.34$, $p = 0.02$). Throughout the year, heterocysts accounted for less than 1% of total cells in the reservoir (data not shown), indicating N_2 fixation contributed very little N to the reservoir.

4. Discussion

The highly-conserved genes for cyanophycin synthesis (*cphA*) and degradation (*cphB*) were confirmed to be present in 21 of 23 ecotypes of *Raphidiopsis. Raphidiopsis raciborskii* QDH7 always commenced to synthesize and accumulate cyanophycin under N-fluctuating conditions, regardless of whether exogenous N was deficient. In the culture experiments, *R. raciborskii* generated cyanophycin through prioritizing uptake of ^{15}N-NaNO$_3$ at N concentrations ≥ 1.2 mg L^{-1}; but cyanophycin-based N was derived from unlabeled N_2 at N concentrations ≤ 1.0 mg L^{-1} (Fig. S7). Cells grown under NO_3^--*N* < 1.0 mg L^{-1} had lower cyanophycin accumulation rates than cells grown under NO_3^--*N* > 1.2 mg L^{-1}. Our field investigation showed that the cyanophycin content remained at a high level in N-sufficient (NO_3^--*N* > 0.45 mg L^{-1}) periods, but decreased in N-deficient summer. In summer, *R. raciborskii* sustained a relative high biomass and formed few heterocysts (< 1%). Our findings strongly suggested that cyanophycin-released N, rather than fixed N, supports the persistent blooms of *R. raciborskii* in N-deficient seasons.

4.1. *Cyanophycin accumulation in R. raciborskii upon N fluctuations, regardless of exogenous N concentration*

Many studies of nutrient storage are biased towards the belief that cyanophycin was accumulated when the exogenous N concentration is high and degraded when it is low (Trautmann et al., 2016; Van de Waal et al., 2010). Several studies, however, have reported an unexplained transient accumulation of cyanophycin in N-starved *Synechocystis* sp. cells (Klotz et al., 2016). Consistent with this, Polerecky et al. (2021) found that the cyanobacterium *Cyanothece* sp. ATCC 51,142 prioritized cyanophycin synthesis under both N-deficient and N-sufficient conditions. These findings suggested that the accumulation of cyanophycin may be independent of absolute N concentrations, and there are likely to be certain factors that trigger its synthesis and degradation. In our culture experiments, N fluctuations triggered cyanophycin synthesis in *R. raciborskii. R. raciborskii* always commenced to synthesize and accumulate cyanophycin when it was inoculated into fresh medium (one type of N fluctuation), or when subjected to simulated N fluctuations in the culture (Fig. 2). As diazotrophic cyanobacteria, unicellular *Cyanothece* sp. ATCC 51,142 and filamentous *Trichodesmium* sp. fix N at night and store the fixed N in the form of cyanophycin (Finzi-Hart et al., 2009; Sherman et al., 1998). Similarly, *R. raciborskii* CS-505 yields distinct cyanophycin polar "nodules" in the heterocysts (Plominsky et al., 2015). Cyanophycin was synthesized at night to store fixed N and degraded during the daytime to mobilize N for photosynthesis in diazotrophic cyanobacteria. These findings led to the belief that diazotrophic cyanobacteria accumulate cyanophycin only during the N_2 fixation process (Watzer and Forchhammer, 2018). However, this does not appear to be the case. Our study revealed that once *R. raciborskii* encountered N fluctuations, it began to accumulate cyanophycin regardless of exogenous N concentration. Therefore, it was suggested that N fluctuations cause *R. raciborskii* to enter an unbalanced growth stage, and the reduction in its growth rate drives the conversion of exogenous N to cyanophycin (Polerecky et al., 2021); whereas during exponential growth, cyanophycin synthesis is bypassed and it is degraded in favor of protein synthesis (Watzer et al., 2015).

4.2. *N threshold determining cyanophycin accumulation or degradation in natural waters*

Previous studies have shown that in natural waters, planktonic algae generally show unbalanced growth in dynamically fluctuating environments (Van de Waal et al., 2010), and *cphA* encoding cyanophycin synthetase is actively transcribed to cope with nutrient fluctuations. Interestingly, *cphB*, which is responsible for cyanophycin degradation, is also very active under such conditions (Tee et al., 2020). These findings suggested that cyanophycin synthesis and degradation co-occurred when *R. raciborskii* encounters N fluctuations; and that the ambient N concentration determines how much cyanophycin is ultimately accumulated or degraded. Our field results supported this idea. We observed that the cellular cyanophycin content was dependent on the exogenous NO_3^--N levels. During N-deficient (< 0.45 mg NO_3^--N•L^{-1}) conditions in summer, cyanophycin was degraded and remained at a relatively low level. In the first half of the year (January to June) when the NO_3^--N concentration was relatively high (average, 0.45 mg TN L^{-1}), the

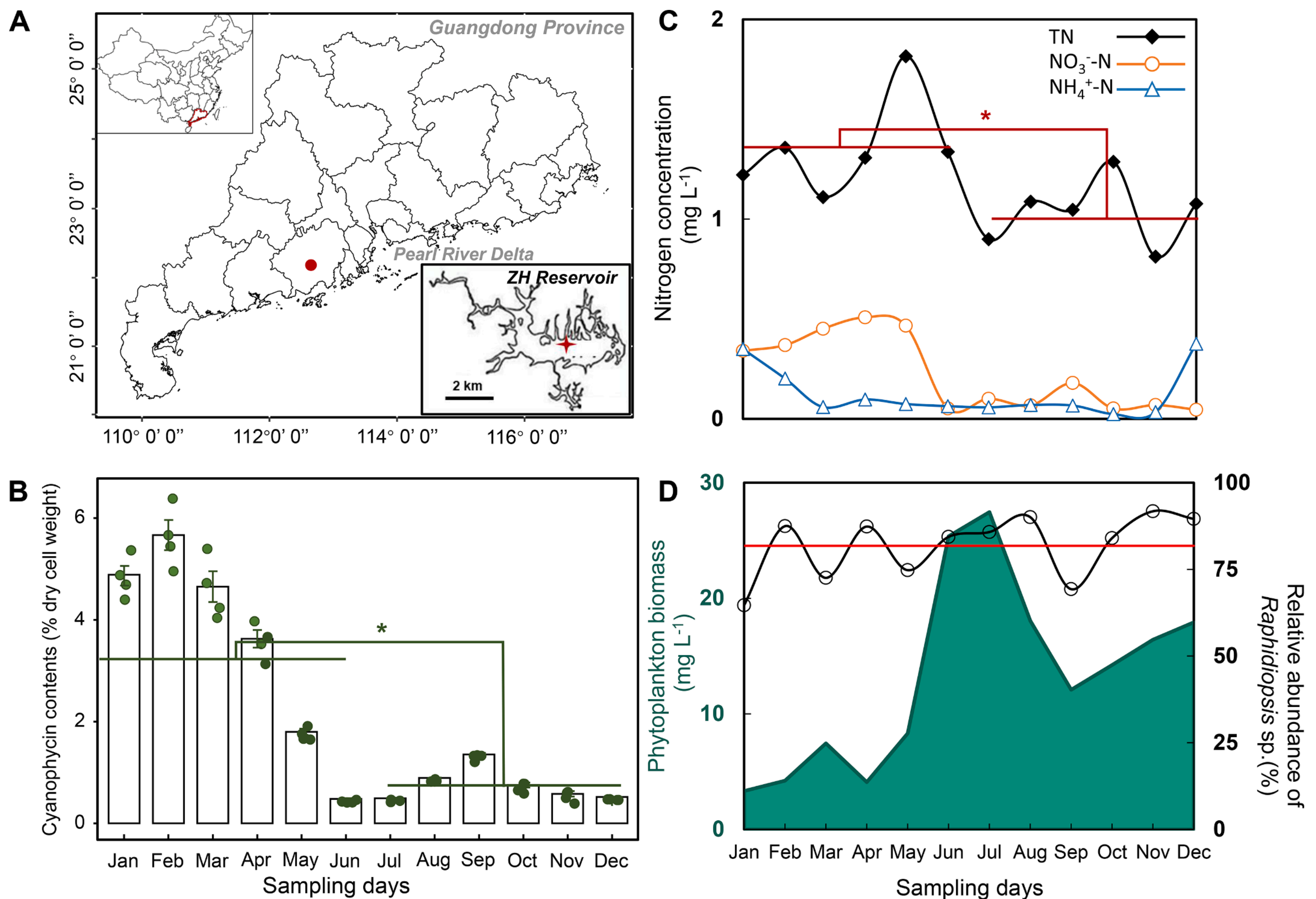

Fig. 4. The monthly changes of the cyanophycin content, N concentration, and phytoplankton biomass in 2019 at ZH Reservoir. (A) The location of study site at ZH Reservoir, located in Guangdong province, China. (B) The cyanophycin contents (% dry cell weight of *Raphidiopsis*). The green lines mean the average of cyanophycin contents in the first half and the second half of the year. (C) The N concentrations, including the TN, NO3–N, and NH4+-N. The red lines mean the average of TNs in the first half and the second half of the year. (D) The phytoplankton biomass (indicated by the area charts, the green color); the relative abundances of *Raphidiopsis*(% of the total phytoplankton biomass, indicated by the white hollow points); the red line means the annual average of relative abundance, that is close to 80%. *, Significance at 0.05 level (paired *t* test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

cyanophycin content was also relatively high (Fig. 4B). Accordingly, we speculated that there exists a threshold N concentration in natural waters that triggers accumulation or degradation of cyanophycin.

The N substrates for cyanophycin synthesis are not only derived from ambient N (Van de Waal et al., 2010), but also from newly fixed N in diazotrophic cyanobacteria (Finzi-Hart et al., 2009). Our ^{15}N-labeling experiments revealed that there was a N threshold determining the source of N in cyanophycin. If algal cells were inoculated into medium containing sufficient NO_3^--N (> 1.2 mg L^{-1}), then ambient $NaNO_3$ was the preferred substrate over N_2. It was suggested that, under a relatively high N concentration, *R. raciborskii* produced cyanophycin as N storage utilizing up-taken available NO_3^--N. If the ambient NO_3^--N concentration was too low (< 1.0 mg L^{-1}) for it to be assimilated into cyanophycin, *R. raciborskii* assimilated an unlabeled external N source, probably as fixed N (as evidenced by the increase in dinitrogenase activity, Fig. 2B). This suggested that N deficiency activated N_2 fixation, and the fixed N, rather than NO_3^--N, likely contributed most of the N substrates for cyanophycin synthesis. The proposed N threshold determining the N_2 fixation behavior could also be found in a field study by González-Madina et al. (2019). They reported that when the TN concentration was higher than 1.0 mg L^{-1}, all the species behaved as non-N_2-fixers (that is, a complete absence of heterocysts, which is an adequate proxy of non-N_2-fixing) (González-Madina et al., 2019). Although *R. raciborskii* could accumulate cyanophycin regardless of exogenous N concentration, once it encountered fluctuating N conditions, cells grown under NO_3^--N < 1.0 mg L^{-1} had lower cyanophycin accumulation rates than cells grown under NO_3^--N > 1.2 mg L^{-1} (Fig. 3). Under high nitrate conditions (NO_3^--N > 1.2 mg L^{-1}), *R. raciborskii* rapidly accumulated cyanophycin on day 1 when the uptake rate of $NaNO_3$ was highest. In contrast, under low nitrate conditions (NO_3^--N < 1.0 mg L^{-1}), a switch from atmospheric N to NH_4^+ caused long lag times in cyanophycin accumulation (Fig. 3A). This is because cells have to produce enzymes first to allow them fix N (Dixon and Kahn, 2004). This finding supports our speculation. There does exist a N threshold in natural waters determining whether cyanophycin is accumulated or degraded, although this natural threshold was lower than that of experimental study due to the complexity of natural waters. When the ambient N concentration was relatively high (NO_3^--N > 0.45 mg L^{-1}), cyanophycin was synthesized faster than it was degraded, leading to cyanophycin accumulation. While the available N concentration became lower than 0.45 mg L^{-1}, the N used for cyanophycin synthesis was derived from fixed N, leading to a long lag in cyanophycin synthesis. Thus, cyanophycin degradation was faster than its synthesis, resulting in decreased cyanophycin content. Based on these findings, we propose that if the ambient N concentration is high enough and fluctuates severely, then *R. raciborskii* will prioritize cyanophycin accumulation to store N by taking up ambient N. When the ambient N concentration becomes low, *R. raciborskii* will first release the N from

recently synthesized cyanophycin to alleviate the N-deficient conditions, thereby minimizing the need for energy-intensive N_2 fixation.

4.3. N stored in cyanophycin accumulated under N-fluctuating and high-N conditions facilitates the persistent dominance and blooms of *R. raciborskii*

As with many other aquatic ecosystems, our studied reservoir showed fluctuations in TN throughout the year. The TN was highest in spring to early summer and declined to low levels as summer progressed (Fig. 4C). It is likely that in summer, the higher temperatures promoted *R. raciborskii* blooms, and their demands for inorganic N depleted the inorganic N to N-limited conditions (Schindler et al., 2008). However, the long duration of the high biomass of *R. raciborskii* from early summer to fall (Fig. 4B) must be supported by sufficient N. Previous studies have also revealed that the rates of N_2 fixation across most freshwater systems are insufficient to alleviate N limitation within the ecosystem (Paerl et al., 2016; Scott and McCarthy, 2010; Shatwell and Köhler, 2019; Xu et al., 2021). Another study showed that N_2 fixation did not confer a competitive advantage upon *R. raciborskii* under N-limiting conditions, and provided little N to support relatively low growth (Willis et al., 2016). Consistent with that finding, we observed few heterocysts ($< 1\%$ of total *R. raciborskii* cells) in our reservoir during the yearlong survey. Meanwhile, the monthly cell quotas (μg Chl-a per μg TP) were greater than 0.3 (Fig. S8), indicating that our studied reservoir was characterized as P-limitation throughout the year (Maberly et al., 2020). P-limitation constrains the synthesis of ATP and related enzymes for N_2 fixation (and/or heterocysts formation) (Stewart and Alexander, 1971; Yema et al., 2016). In addition, we isolated two strains from this reservoir and found that under N-depleted conditions, these two strains produced a low proportion of heterocysts (Fig. S9). Heterocysts are specialized cells to fix N (Flores and Herrero, 2010), and so the presence of few heterocysts is indicative of rather low inputs of fixed N. Also, *R. raciborskii* is not a surface bloom former, but rather is distributed throughout the water column, where light (energy) limitation may constrain the ability to fix N_2. Furthermore, internal NH_4^+-N regenerated from sediments was reported to help sustain cyanobacterial blooms in Lake Taihu (Xu et al., 2021). Unlike shallow lake, however, the ZH reservoir we studied has a maximum depth of 12 m. The depth makes the hypolimnion seasonally undisturbed by hydrodynamic actions with only the epilimnion staying active (Qin et al., 2020). Thus, we suggest that regenerated NH_4^+-N may not meet the N demand for summer-fall, *R. raciborskii*-dominated blooms in our studied system. These findings and those of previous reports illustrate that another N source must be supporting the persistent dominance and blooms of *R. raciborskii* in N-deficient summer waters.

Just as analogous to polyP, this effective P storage allows cyanobacteria to take up more P than is required in P-sufficient conditions so that cells can continue to grow later under P-deficient conditions (Willis et al., 2017). Similarly, we found that under N-sufficient condition in spring, the cyanophycin content in *R. raciborskii* was relatively higher. Thus, we propose that *R. raciborskii* also assimilated more N than that was required for cell growth under N-sufficient conditions in spring. As for the function of this stored N, the rationale may be similar to that of polyP, which extends the duration in which *R. raciborskii* can sustain growth under P deficiency (Xiao et al., 2020). The polyP quota generally decreased and supported high primary productivity in the P-deficient season in Lake Ontario (Li et al., 2019). There are two main N storages, cyanophycin and phycobilisomes in cyanobacteria. Compared to cyanophycin, phycobilisomes has a dual role in N storage and photosynthesis (Forchhammer and Schwarz, 2019). The degradation of phycobilisomes changes the water color from blue-green to yellow and appear chlorotic (Allen et al., 1980; Simon, 1971). During our observation, the water color was basically unchanged, remaining blue-green the year round. We thus suggest that cyanophycin, not phycobilisomes was used as the main internal N storage for the diazotrophic *R. raciborskii* (Li et al., 2001). We found that the cyanophycin dynamics were diametrically opposite to the population dynamics of *R. raciborskii* in the ZH Reservoir (Fig. 4B and 4D). Integrating our findings and the previous reports, we develop a potential scenario for the function of cyanophycin. In the high-N (NO_3^--N > 0.45 mg L^{-1}) season, *R. raciborskii* preferentially activates cyanophycin accumulation; when the NO_3^--N concentration declines (< 0.45 mg L^{-1}), and it prioritizes cyanophycin degradation, releasing internal N to alleviate the cells from N deficiency. Especially under light energy limited conditions (i.e., in subsurface populations), this flexible N storage and rapid release capability likely obviates the need for the energy-intensive N_2 fixation, allowing *R. raciborskii* to proliferate and sustain its dominance during N-deficient seasons. Although the threshold of N concentration might be approximate, it provides some insights into the mechanism by which *R. raciborskii* can proliferate under N-deficient conditions. We therefore suggest that as nutrient storage molecules, polyP and cyanophycin may jointly facilitate the dominance of cyanobacteria in environments with fluctuating nutrient availability. These mechanisms allow bloom-forming cyanobacteria to avoid nutrient limitation.

5. Conclusion

Our study verified the presence of cyanophycin associated genes and granules in *R. raciborskii*. Our experimental strain (QDH7 of the species) always commenced to accumulate cyanophycin under simulated fluctuating N conditions. We suggested that there is a NO_3^--N threshold ($>$ 0.45 mg L^{-1}) determining whether cyanophycin is accumulated or degraded in natural waters. The cyanophycin content was closely associated with the population dynamics of *R. raciborskii* in a large tropical reservoir (ZH Reservoir, southern China), indicating that N released from stored N (in the form of cyanophycin), rather than fixed N, supported the proliferation of *R. raciborskii* under N-deficient summer conditions. Our study demonstrated the role of stored N in cyanobacterial dominance and bloom formation. Due to this highly adaptive strategy in a N_2-fixing cyanobacterial species, mitigating its bloom will be more difficult than previously assumed.

CRediT authorship contribution statement

Zhe Lu: Conceptualization, Methodology, Data curation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Jinmei Ye:** Methodology, Investigation. **Zhijiang Chen:** Methodology, Investigation. **Lijuan Xiao:** Methodology, Investigation. **Lamei Lei:** Conceptualization, Resources, Writing – original draft, Funding acquisition. **Bo-ping Han:** Writing – review & editing. **Hans W. Paerl:** Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2022.118215.

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