Elevated temperature relieves phosphorus limitation of marine unicellular diazotrophic cyanobacteria

Lixia Deng,1,2 Shunyan Cheung,2* Chang-Keun Kang,4 Kailin Liu,2 Xiaomin Xia,5 Hongbin Liu2,3,6*

1Thrust of Earth, Ocean and Atmospheric Sciences, The Hong Kong University of Science and Technology, Guangzhou, China
2Department of Ocean Science, The Hong Kong University of Science and Technology, Hong Kong, China
3Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou), Guangzhou, China
4School of Earth Science and Environmental Engineering, Gwangju Institute of Science and Technology, Gwangju, Republic of Korea
5Key Laboratory of Tropical Marine Bio-Resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, China
6Hong Kong Branch of Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou), The Hong Kong University of Science and Technology, Hong Kong, China

Abstract

The warming ocean is expected to be more phosphorus (P) limited due to increasing stratification. P is a major limiting nutrient of marine diazotrophs, while the interactive effect of temperature elevation and P limitation on marine unicellular diazotrophs is unknown. Here, we examined the physiology of a major unicellular diazotroph, Crocosphaera watsonii, grown under P-limited and P-replete conditions at 25°C, 28°C, and 31°C. Growth, N2, and CO2 fixation rates of C. watsonii increased with temperature under P limitation, and growth rates were similar between P-limited and P-replete treatments at 31°C. At high temperature, the P use efficiencies for N2 and CO2 fixation under P limitation were more than twice higher than under P-replete conditions. Expression of genes involved in P acquisition, intracellular recycling, and substitution in C. watsonii was upregulated at higher temperature under P limitation. These results suggest that P limitation in C. watsonii was relieved with elevated temperature through various temperature-dependent economic strategies on P metabolism. Through meta-analysis of a field data set using general additive model, we found that C. watsonii abundance was correlated mainly with temperature and phosphate, and predicted to increase significantly with further warming.

Nitrogen (N)-fixing (diazotrophic) cyanobacteria play a crucial role in marine biogeochemical cycles, particularly in oligotrophic waters, where diazotrophic cyanobacteria are the major new N source of primary productivity and they mediate carbon (C) and N export of the euphotic zone (Karl et al. 1997; Mulholland 2007). Trichodesmium is the most well-studied diazotrophic cyanobacterium, which has long been regarded as a dominant N2 fixer in the ocean (Capone et al. 1997; Bergman et al. 2013; Zehr and Capone 2020). However, diverse unicellular cyanobacteria were later discovered abundant in tropical and subtropical oceans and conducting equal or more N2 fixation compared with Trichodesmium (Moisander et al. 2010; Martínez-Pérez et al. 2016). Marine unicellular diazotrophic cyanobacteria consist of three phylogenetically distinct groups, including UCYN-A, UCYN-B, and UCYN-C (Zehr 2011). So far, UCYN-B (Crocosphaera watsonii) and UCYN-C (Cyanothece) have been cultivated (Webb et al. 2009; Taniuchi et al. 2012), but UCYN-B is more abundant than UCYN-C in the open ocean (Shiozaki et al. 2017; Chen et al. 2019).

C. watsonii has been frequently used as a major model organism for understanding the physiology of marine unicellular diazotrophic cyanobacteria (Fu et al. 2014; Masuda et al. 2018; Yamaguchi et al. 2020). To date, culture-based experiments have shown that a number of environmental factors appear to affect the physiology of C. watsonii, such as

*Correspondence: liuhb@ust.hk; sycheungab@connect.ust.hk

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temperature and nutrient availability, especially phosphorus (P) and iron (Fe) (Fu et al. 2014; Zhu et al. 2020; Yang et al. 2021). The physiological rates of C. watsonii showed unimodal thermal response curves, with the optimal temperatures for growth, CO₂ and N₂ fixation ranging 28–30°C (Fu et al. 2014). Growth and N₂ fixation of C. watsonii can be limited by either P (Zhu et al. 2020), or Fe (Yang et al. 2021), as well as the combination of the two (Garcia et al. 2015).

Global warming is projected to lead to about a 3°C increase in the mean sea surface temperature by the end of this century due to the high anthropogenic CO₂ emission (Collins et al. 2013). In particular, ocean warming for the surface waters will be the strongest in the tropical and subtropical regions (Stocker et al. 2013), where the major domains of the marine diazotrophic cyanobacteria are found (Cheung et al. 2020; Zehr and Capone 2020). The increase in sea surface temperature will intensify the stratification of surface ocean and subsequently reduce the replenishment of nutrients (e.g., nitrate and phosphate) from the subsurface water (Doney 2006). The decline of N supply is proposed to limit nondiazotrophic phytoplankton growth but select diazotrophic cyanobacteria that can fix N₂ (Karl et al. 2001). Thus, diazotrophic cyanobacteria may experience more P-impoverished condition in the warming ocean (Hutchins and Fu 2017), especially in oligotrophic waters, where P is mainly delivered to the surface water from deeper water via mixing and advective processes (Karl 2014). P is crucial to the growth and metabolism of organisms since it is a key element in nucleic acids, phospholipids and other important biomolecules (Karl 2014). Many studies have suggested that P limits N₂ fixation by diazotrophs in marine ecosystems (Webb et al. 2007; Sohm et al. 2008; Turk-Kubo et al. 2012). In contrast, atmospheric Fe input into the surface ocean was proposed to increase under the global climate change (Hutchins and Boyd 2016). Hence, marine diazotrophs are expected to face both temperature elevation and more severe P limitation in the projected warming ocean, whereas how they respond to the interaction of temperature elevation and P limitation remains obscure. Considering the ecological importance of the diazotrophic cyanobacteria in supporting the fertility, productivity, and C sequestration of the ocean (Zehr and Capone 2020), it is important to understand the physiological responses of marine diazotrophic cyanobacteria under the proposed conditions in the future ocean.

So far, studies of interactive effects of temperature and P availability on marine diazotrophic cyanobacteria were only limited to *Trichodesmium*, in which different *Trichodesmium* strains showed inconsistent responses to warming under P-limited conditions (Hutchins et al. 2007; Qu et al. 2019). It should be noted that C. watsonii and *Trichodesmium* have different growth thermal limits and optimum growth temperatures (Fu et al. 2014). These two N₂ fixers also differ in P metabolism. In contrast to *Trichodesmium*, C. watsonii is unable to utilize phosphonates, which roughly account for 25% of the dissolved organic P in the ocean (Clark et al. 1998; Pereira et al. 2019). In addition, due to its smaller cell size, C. watsonii has larger cell surface area to volume ratio for the uptake of P compared to *Trichodesmium* (Finkel et al. 2009). Therefore, investigating the physiological responses of unicellular diazotrophic cyanobacteria to temperature elevation and P limitation is in critical need. In addition, none of the previous relevant studies has studied the underlying molecular mechanisms (Hutchins et al. 2007; Qu et al. 2019). Various strategies for overcoming P limitation have been proposed in cyanobacteria (Pereira et al. 2019), including high-affinity scavenging for extracellular inorganic phosphate, utilizing organic P compounds, and lowering cellular P requirement by element substitution (Dyhrman and Haley 2006; Van Mooy et al. 2006; Pereira et al. 2019). It has also been reported that C. watsonii has versatile genetic potential of P acquisitions (Pereira et al. 2019). However, it is uncertain whether/how temperature would affect the processes of P metabolism and hence mediate the physiological responses of C. watsonii to P limitation in a warming ocean.

In order to better predict the fate of unicellular diazotrophic cyanobacteria as well as N₂ fixation in the future climate scenario, the response of C. watsonii to the interactive effects of temperature elevation and P limitation was resolved with both laboratory experiments and a meta-analysis of a field observational data set in the current study. We examined the physiological responses including growth and N₂ and CO₂ fixation rates, as well as the molecular responses (i.e., the expression of P metabolism related genes) of C. watsonii under P-limited (200 nmol L⁻¹) and P-replete (45 μmol L⁻¹) conditions at three temperatures (25°C, 28°C, and 31°C). Besides, a meta-analysis on a global data set of C. watsonii abundance was also conducted to provide additional evidence of the effects of temperature and P availability on C. watsonii in the real ocean.

**Materials**

**Cell culturing and experimental design**

C. watsonii WH8501 cultures were maintained in N-free Scripps Oceanographic medium (Tuit et al. 2004), prepared with 0.2 μm filtered artificial seawater and 36 μmol L⁻¹ Na₂HPO₄. Cultures were grown in polycarbonate bottles at the light intensity of 80 μmol photons m⁻² s⁻¹ with a 12 : 12-h light : dark cycle. Cells were acclimated at three temperatures (25°C, 28°C, and 31°C) and diluted with fresh medium every 2 weeks for at least 6 months. To acclimate the cultures to P-limited conditions, we transferred half volume of cultures to the equal volume of fresh P-limited (200 nmol L⁻¹ phosphate) medium every 3 d. After repeating that for a number of times and the culture reached the targeted phosphate concentration, each bottle of culture was diluted every 3–4 d to the initial cell density of around 5 × 10⁴ cells mL⁻¹ with fresh P-limited medium. The cell abundance was checked using a Becton–
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N2 and CO2 fixation rates measurement

N2 and CO2 fixation rates were determined using the 15N2 gas tracer and 13C-tracer addition method, respectively (Hama et al. 1983; Montoya et al. 1996; Mulholland and Berhardt 2005). Briefly, 3 h before the onset of the dark period, each 60-mL serum bottle was filled with culture samples from a treatment replicate but leaving some headspace and 64 μL of 0.2 mol L⁻¹ NaH13CO3 (Sigma-Aldrich) was added to each bottle. Serum bottles were then capped with Telfon-coated butyl rubber septum caps, 0.5 mL of 15N2 gas (Cambridge Isotope Laboratories) was injected into each serum bottle with a gas-tight syringe. All the samples were incubated for 24 h at designated temperatures. After incubations, the samples were filtered onto precombusted (550°C, 5 h) GF/A filters (Whatman) and stored at –80°C before analysis using wet oxidation method (Pujo-Pay and Raimbault 1994).

Phosphorus use efficiency

The phosphorus use efficiencies (PUEs) for N2 and CO2 fixation were defined as the quantity of N2 or CO2 fixed per unit time per unit cellular P, respectively (Qu et al. 2019). In the calculation of N-specific and C-specific PUEs, N2 and CO2 fixation rates were normalized to cellular particulate organic P, respectively.

Cell size measurement

The cell size of C. watsonii WH8501 at each treatment was measured by the microscopic observation. To avoid the effects of diel variation in cell size of C. watsonii (Mohr et al. 2010), samples from different treatments were collected simultaneously. Samples (1 mL) collected from each treatment were fixed with 20% paraformaldehyde solution (Guo et al. 2014), and preserved at –80°C until analysis with a Becton–Dickinson FACSCalibur flow cytometer.

Quantifying expression levels of the genes involved in P metabolism

Given that the expression of the genes involved in P metabolism in C. watsonii exhibited a diel pattern (Shi et al. 2010), RNA samples were collected from each treatment replicate in the middle of light and dark period, respectively. The samples (50 mL) were filtered onto 1-μm pore size polycarbonate filter membranes under low vacuum pressure. Each membrane sample was then placed in a sterilized centrifuge tube and immediately stored at –80°C after adding 1 mL TRIzol Reagent (Ambion, Life Technologies). RNA was extracted using the TRIzol Reagent and PureLink RNA Mini Kit (Ambion, Life Technologies) and subsequently reverse-transcribed into cDNA using HiScript® III RT SuperMix for quantitative polymerase chain reaction (qPCR) with gDNA wiper (Vazyme) according to the manufacturers' protocols.

Relative transcript abundances of the genes involved in P metabolism were quantified using qPCR assays with the primers designed in a previous study (Pereira et al. 2016). We examined the transcription of 10 P metabolism related genes that involved in gene regulation (phoB, phoU, and phoH), P scavenging (pstS, sphX, and phoA), substitution (sqdB), and recycling (ppX, ppK, and ppA) in response to P limitation at different temperatures. Each qPCR reaction was run with SYBR Green SuperMix (Roche Diagnostics GmbH), containing 5 μL...
SYBR Green SuperMix, 1 μL forward and reverse primers (10 pmol/μL), 3.5 μL ultrapure distilled water (Invitrogen, Thermo Fisher Scientific Corp.), and 0.5 μL cDNA. The reactions were run with triplicates in 384-well plates on a Light Cycler 480 (Roche Diagnostics GmbH) with the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. No-template controls were run in triplicates during each qPCR. The $2^{-\Delta\Delta C_t}$ method was used to normalize the expression level of each gene (Livak and Schmittgen 2001). The threshold cycle (Ct) value of each gene was normalized to a housekeeping gene (mpB, encoding the RNA component of RNase P), whose transcript abundance was found not to vary under different nutrient and temperature conditions in marine cyanobacteria (Gómez-Baena et al. 2009; Chappell and Webb 2010). A previous study also showed that the transcription of rnpA gene (encoding the protein component of RNase P) in C. watsonii WH8501 does not show a diel variation (Shi et al. 2010).

Analyzing the nifH gene abundance of C. watsonii in field using general additive model

We used general additive model (GAM) to analyze the relationship between the nifH gene abundance of C. watsonii and environmental factors in the global ocean using a data set compiled by Tang and Cassar (2019). This data set contains nifH gene abundance of C. watsonii estimated by qPCR using the DNA samples collected from the Pacific Ocean, Atlantic Ocean, Indian Ocean, and their marginal seas, together with the corresponding environmental factors (i.e., temperature, phosphate, nitrate, and nitrate to phosphate ratio) (Tang and Cassar 2019). The environmental factors that showed significant relationships ($p < 0.05$) with the nifH gene abundance of C. watsonii were selected as the predictors for further constructing a GAM to predict the nifH gene abundance of C. watsonii. The data set of C. watsonii nifH gene abundance was partitioned randomly into training (90%) and testing (10%) data sets using package caTools in R (Tuszynski 2012). The training data set was used to construct the model. The environmental factors in the testing data set were input into the model to predict the corresponding C. watsonii nifH gene abundance. The observed and predicted values of the C. watsonii nifH gene abundance of the testing data set were compared to evaluate the predictive accuracy of the model. To estimate the changes of C. watsonii nifH gene abundance under warming and P limitation, we predicted C. watsonii nifH gene abundance with altered environmental predictors using this model, in which the temperature and phosphate concentration were increased by 4°C and reduced by five times, respectively. The GAM analysis was conducted using the package “mgcv” in R (Wood and Wood 2015).

Statistical analysis

Two-way ANOVA was used to test the significance of the differences in the interactive effects of temperature and phosphate concentration on C. watsonii, and one-way ANOVA was applied to test the significance of the differences for both P levels among different temperatures. The Tukey multiple comparison test was used to determine the significance of the differences between individual treatments. All statistical analyses were performed with a SPSS 26.0 software. The significance level of 0.05 was set.

Results

Growth rates and cell size of C. watsonii

Growth rates of C. watsonii WH8501 under P-limited conditions were significantly lower than under P-replete conditions regardless of the temperature (two-way ANOVA, $p < 0.001$), indicating that the low phosphate concentration inhibited the growth of C. watsonii (Fig. 1A). Under P-replete conditions, growth rates of C. watsonii at 31°C were significantly lower

Fig. 1. (A) Growth rates (d$^{-1}$) and (B) cell size (μm) of Crocosphaera watsonii WH8501 under P-limited and P-replete conditions (200 nmol L$^{-1}$ and 45 μmol L$^{-1}$) at three temperatures (25°C, 28°C, and 31°C). Given morphological data (e.g., cell size) were generally more variable than the physiological data (e.g., growth rates), cell diameters of C. watsonii were shown as a box plot. Values denote mean ± standard deviation of biological replicates under each treatment ($n = 3$ for growth rates, $n ≥ 15$ for cell size).
than at 25°C and 28°C ($p < 0.05$), with the highest growth rates at 28°C (Fig. 1A). In contrast, the growth rates of *C. watsonii* increased with rising temperature (i.e., from 25°C to 31°C) under P-limited conditions (Fig. 1A). Meanwhile, the differences among the growth rates under P-limited and P-replete treatments decreased with increasing temperature. Specifically, P limitation lowered the growth rates of *C. watsonii* by 35% at 25°C, 21% at 28°C and 6% at 31°C, which suggested that elevated temperature lessened the effects of P limitation and almost relieved P limitation on the growth of *C. watsonii* at 31°C (Fig. 1A).

Both the temperature (two-way ANOVA, $p < 0.01$) and P availability (two-way ANOVA, $p < 0.01$) had a significant effect on cell diameters ($\mu$m) of *C. watsonii*. Increased temperature resulted in a decrease of cell size in the P-limited cultures, with the cell diameters decreasing from 25°C to 28°C by 23% and to 31°C by 33% (Fig. 1B). However, the cell diameters in the P-replete cultures did not vary significantly among different temperatures ($p > 0.05$, Fig. 1B). At 25°C, the cell diameters in the P-replete cultures were much larger than those in the P-replete cultures ($p < 0.05$, Fig. 1B), while there was no significant difference between these two P levels at 28°C and 31°C ($p > 0.05$, Fig. 1B).

### N$_2$ and CO$_2$ fixation rates

Both temperature (two-way ANOVA, $p < 0.01$) and P availability (two-way ANOVA, $p < 0.05$) significantly affected N$_2$ fixation rates of *C. watsonii*. Under P-limited and P-replete conditions, elevated temperature resulted in an increase in N$_2$ fixation rates, but no significant difference was observed among different temperatures under P-replete conditions ($p > 0.05$, Fig. 2A). In contrast, under P-limited conditions, N$_2$ fixation rates did not change significantly from 25°C to 28°C ($p > 0.05$) and increased by 246% from 25°C to 31°C ($p < 0.05$, Fig. 2A). Thus, as with growth rates, elevated temperature greatly enhanced the N$_2$ fixation capabilities of *C. watsonii* under P-limited conditions.

CO$_2$ fixation rates were also affected by temperature (two-way ANOVA, $p < 0.01$) and P availability (two-way ANOVA, $p < 0.001$). Under P-limited conditions, CO$_2$ fixation rates increased greatly by 495% from 25°C to 31°C ($p < 0.05$), though the difference between 28°C and 31°C was not significant ($p > 0.05$, Fig. 2B). Under P-replete conditions, CO$_2$ fixation rates reached a maximum at 28°C, significantly higher than at 25°C and 31°C ($p < 0.05$, Fig. 2B). Hence, elevated temperature also had a stronger positive effect on the CO$_2$ fixation capabilities of *C. watsonii* under P-limited conditions.

![Fig. 2.](image-url) (A) N$_2$ fixation rates (ng N mg$^{-1}$ h$^{-1}$), (B) CO$_2$ fixation rates (ng C mg$^{-1}$ h$^{-1}$), (C) N-specific PUE (ng N h$^{-1}$ $\mu$g P$^{-1}$), (D) C-specific PUE (ng C h$^{-1}$ $\mu$g P$^{-1}$) of *Crocosphaera watsonii* WH8501 under P-limited and P-replete conditions (200 nmol L$^{-1}$ and 45 $\mu$mol L$^{-1}$) at three temperatures (25°C, 28°C, and 31°C). Values denote mean ± standard deviation of biological replicates under each treatment ($n = 3$).
than under P-replete conditions. The differences of CO₂ fixation rates between P-limited and P-replete cultures were larger than those of N₂ fixation rates (Fig. 2A,B).

**Phosphorus use efficiency**

The PUE for N₂ fixation (ng N h⁻¹ μg⁻¹ P) under P limitation was higher than under P-replete conditions regardless of the temperature (two-way ANOVA, p < 0.05, Fig. 2C). Under P limitation, the PUE for N₂ fixation increased slightly from 25°C to 28°C (p > 0.05) and increased significantly by 144% from 25°C to 31°C (p < 0.05, Fig. 2C). Under P-replete conditions, the PUE for N₂ fixation increased slightly with rising temperature, although no significant difference was observed among different temperatures (p > 0.05, Fig. 2C). For both N₂ and CO₂ fixation, the PUEs showed thermal response patterns similar to the corresponding rates (Fig. 2).

**Expression levels of P metabolism genes**

Comparing with the P-replete cultures, the genes related to P scavenging (pstS) and the associated regulator (phoU) were significantly upregulated in the P-limited cultures at all tested temperatures (p < 0.05, Fig. 3A). Specifically, the expression of pstS gene increased 3- to 13-fold under P-limited conditions, while the expression levels of phoU gene in the P-limited cultures were about 4-fold higher than in the P-replete cultures (Fig. 3A). The expression of another P scavenging gene (sphX) increased significantly in the P-limited cultures compared to the P-replete cultures at high temperature (i.e., threefold at 28°C and eightfold at 31°C) (p < 0.05, Fig. 3A). The genes involved in degradation of polyphosphate (polyP) (ppX) and regulation of phosphate transport (phoB) were only upregulated in the P-limited cultures at 31°C (p < 0.05, Fig. 3A).

To explore at the molecular level how *C. watsonii* responds to ocean warming under P limitation, we compared the expression levels of the genes involved in P metabolism with rising temperature under P-limited and P-replete conditions (Figs. 3B,C). With increasing temperature, P-limited *C. watsonii* significantly upregulated several genes involved in P scavenging (pstS and sphX) and substitution (sqdB), gene regulation (phoB and phoH), and intracellular P recycling (ppA and ppK) (p < 0.05, Fig. 3B). The expression levels of these genes were twofold to fourfold higher at 28°C and 31°C than at 25°C under P limitation (Fig. 3B). The expression of the genes involved in regulating the phosphate transport (phoU) and hydrolyzing dissolved organic P (phoA) did not change significantly with increasing temperature under P limitation (p > 0.05, Fig. 3B). Under P-replete conditions, ppA gene was significantly upregulated with increasing temperature.

![Fig. 3.](image-url)
(p < 0.05, Fig. 3C), while the genes involved in P scavenging (pstS and sphX), polyP regeneration (ppX and ppK) and transcriptional regulation (phoB) were downregulated with increasing temperature (p < 0.05, Fig. 3C).

Environmental determinants and prediction of the nifH gene abundance of *C. watsonii* in field

Among all the tested physicochemical factors, temperature and phosphate showed significant relationships with the nifH gene abundance of *C. watsonii* as revealed by the GAM, in which these two factors explained 73.2% variation of the nifH gene abundance of *C. watsonii*. Temperature showed a positive and strong relationship with the nifH gene abundance of *C. watsonii* (p value < 2 × 10^{-16}), while phosphate showed a slightly negative correlation with the nifH gene abundance of *C. watsonii* (p value = 0.0086) (Figs. 4A,B). These two factors were further used for constructing a GAM (C. watsonii nifH gene abundance ~ s [temperature] + s [phosphate]) that was used to predict the nifH gene abundance of *C. watsonii* under the ambient, high temperature (temperature + 4°C; phosphate concentration unchanged) and high temperature and low phosphate (temperature + 4°C; phosphate concentration reduced by five times) conditions. The predicted nifH gene abundance of *C. watsonii* with the ambient temperature and phosphate concentration was similar to the observed nifH gene abundance in both training (r² = 0.72) and testing (r² = 0.66) data sets. When the temperature was increased by 4°C, the average nifH gene abundance of *C. watsonii* was predicted to increase from 10^7 to 10^8 nifH gene copies m^{-3} (Fig. 4C). Under the high temperature conditions (+ 4°C), P limitation was predicted to slightly increase the nifH gene abundance of *C. watsonii* (Fig. 4C).

Discussion

Interactive effects of warming and P limitation on the physiological rates of *C. watsonii*

As revealed by the incubation experiments, P limitation had a negative impact on growth, N₂ and CO₂ fixation rates of *C. watsonii* in general (Figs. 1, 2), which agreed with previous studies (Garcia et al. 2013b; Zhu et al. 2020). However, we found that these physiological rates of *C. watsonii* increased with temperature under P limitation. As such, the growth rates of *C. watsonii* under P-limited and P-replete conditions became similar at 31°C (Fig. 1A). Moreover, the PUEs for N₂ and CO₂ fixation in *C. watsonii* increased with temperature under P limitation (Fig. 2C,D). These results suggested that the impact of P limitation on *C. watsonii* is temperature dependent and high temperature (31°C) can relieve the P limitation of *C. watsonii*. Similarly, warming was also found to promote growth, CO₂
and N₂ fixation rates of *Trichodesmium erythraeum* GBRTRLI101 under P limitation (Qu et al. 2019), despite many physiological differences between *Trichodesmium* and *C. watsonii* (e.g., optimal growth temperature, thermal range, P metabolism, and cell size). Besides, although growth rates were similar between P-limited and P-replete cultures at high temperature (Fig. 1A), CO₂ fixation rates were still low in P-limited cultures relative to the other physiological rates at high temperature (Fig. 2B). These results suggested that growth and CO₂ fixation may not be closely coupled in *C. watsonii* in response to P limitation. In other words, *C. watsonii* does not require as much C as it fixes under P-replete conditions for survival. It has been found that *C. watsonii* formed carbohydrate granules for storing excessively fixed C under nutrient sufficient conditions (Dron et al. 2012). In contrast, the inhibitory effect of P limitation on N₂ fixation was positively correlated with temperature (Fig. 4). Nevertheless, some differences in the thermal responses of *C. watsonii* were observed between these two studies. For example, less, some differences in the thermal responses of was positively correlated with temperature (Fig. 4). Nevertheless, some differences in the thermal responses of *C. watsonii* were observed between these two studies. For example, less, some differences in the thermal responses of

In addition, warming increased PUEs and Fe use efficiencies (Yang et al. 2021). All these results collectively explained why diazotrophic cyanobacteria are thriving in warm and oligotrophic waters (Sohm et al. 2011) and implied that diazotrophic cyanobacteria could be selected and flourishing in a future warmer ocean. It is projected that *Trichodesmium* and *C. watsonii* could increase their N₂ fixation rates by ~22% and ~91%, respectively, from 2010 to 2100 under the IPCC RCP 8.5 warming scenario, thereby increasing the relative importance of *C. watsonii* as new N source in the future warming of Fe-limited ocean (Jiang et al. 2018; Yang et al. 2021). Additional research efforts are needed to predict the responses of N₂ fixation rates of different diazotrophic cyanobacteria to P-limited and even Fe and P co-limited conditions in the warming ocean.

**Molecular mechanisms for relieving P limitation in response to warming**

As revealed by qPCR analysis, the expression of the genes involved in P scavenging and metabolism was influenced by both P availability and temperature (Fig. 3), suggesting the potential underlying mechanisms that led to the alleviation of P limitation of *C. watsonii* under elevated temperature (Fig. 5). Under P limitation, the expression of high-affinity phosphate transporters (*pstS* and *sphX*) and the associated transcriptional regulator (*phoB*) was upregulated as temperature increased (Figs. 3, 5). The *phoB* regulates transcription of the genes encoding high-affinity phosphate transporters in response to P availability (Wanner 1993; Reistetter et al. 2013). Hence, our result implied that elevated temperature increases the dissolved inorganic P uptake through high-affinity phosphate transporters (*pstS* and *sphX*) and associated transcriptional regulator (*phoB*) under P limitation (Fig. 5). Besides, the transcription of *sqdB* gene that encodes sulfolipid biosynthesis protein was upregulated with temperature under P-limited conditions (Figs. 3, 5). Sulfolipid was reported to substitute the P-containing phospholipid in cyanobacteria under P-limited conditions (Van Mooy et al. 2009). Although our results showed that P limitation did not upregulate the expression of *sqdB* gene in *C. watsonii* at normal growing temperature, which was consistent with previous studies (Van Mooy et al. 2009; Pereira et al. 2016), rising temperature can boost up the expression of *sqdB* gene under P limitation (Fig. 3). *C. watsonii* could reduce their P requirement by enhancing the
Fig. 5. Schematic illustration showing the regulation of P metabolism genes in C. watsonii WH8501 in response to warming under (A) P-limited (200 nmol L$^{-1}$) and (B) P-replete conditions (45 μmol L$^{-1}$). We suggest that enhanced P acquisition, substitution, and intracellular cycling help to relieve P limitation at high temperature. Colors at the periphery of the gene pictograms denote different P metabolisms, and those in the center denote upregulation or downregulation of the P metabolism genes.
replacement of sulfolipid for phospholipid under higher temperatures (Fig. 5). Moreover, the transcription of the genes for intracellular P recycling (ppK and ppA) increased with rising temperature under P limitation (Figs. 3, 5). In cyanobacteria, the polyP kinase ppK can hydrolyze ATP into polyP (serves as cellular phosphate pool) as well as form ATP from polyP (Lin et al. 2016). The ppA gene (encoding inorganic pyrophosphatase) is responsible for the pyrophosphate hydrolysis (Gómez-García et al. 2003). The upregulation of ppK and ppA suggested that the increasing utilization and recycling of intracellular P may be a key strategy for C. watsonii to cope with P stress at high temperature (Fig. 5). In contrast, Pscavenging (psdS, sphX, and phoB) and polyP metabolism genes (ppX and ppK) were downregulated with increasing temperature under P-replete conditions (Figs. 3, 5). It could be because that C. watsonii does not need to enhance P scavenging and metabolism under P-replete conditions.

The growth rate hypothesis proposes that organisms with higher growth rates require greater P rich ribosomal RNA to sustain rapid protein synthesis rates (Elser et al. 1996; Sterner and Elser 2002; Gillooly et al. 2005). According to the growth rate hypothesis, the P requirement of fast-growing organisms is expected to be increased. Higher growth rates under high temperature would lead to stronger P limitation of the organisms. However, a later hypothesis (the temperature-dependent physiology hypothesis; Toseland et al. 2013; Yvon-Durocher et al. 2015) proposes that the cellular P requirement and metabolism are also influenced by temperature. In the current study, our results suggested that P acquisition, substitution, and intracellular cycling in C. watsonii were upregulated by warming under P-limited conditions (Fig. 5), which explained why PUEs of C. watsonii increased and hence P limitation was relieved at high temperature.

Implications for C. watsonii in future ocean

Our study found that P limitation on the physiological rates of C. watsonii is relieved at elevated temperature. We further proposed, based on the transcription of genes involved in P metabolism, that C. watsonii evolved multiple temperature-dependent strategies to overcome P limitation in the ocean (Fig. 5). Analysis of existing field data by the GAM revealed that the nifH gene abundance of C. watsonii in global ocean was positively correlated with temperature and slightly negatively correlated with phosphate (Figs. 4A,B), indicating that C. watsonii is more abundant under warm and P-depleted conditions. We then used the GAM to estimate that the nifH gene abundance of C. watsonii will increase significantly with further increasing temperature and P limitation (Fig. 4C). Hence, both laboratorial experiments and meta-analysis of field observations implied that C. watsonii will thrive in the projected warmer and P-limited ocean. Moreover, in the context of global climate change, other major environmental stresses like elevated pCO2 and increasing light intensities have also been found to enhance the growth and CO2 and N2 fixation of C. watsonii (Garcia et al. 2013a; Gradoville et al. 2014). All these findings lead to a reasonable hypothesis that C. watsonii will be selected and successful in the future ocean. In addition, under P-limited conditions, elevated temperature resulted in the decrease of the cell size of C. watsonii, which may alter its sinking efficiency and hence the C and N export in the future ocean (Fig. 1B). The interactive effects of multiple environmental factors associated with climate change on C. watsonii, and the ecological consequences of physiological changes of C. watsonii to the community structure, trophic interaction and C and N cycling of the microbial food web in the future warming ocean should be further explored.

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Conflict of Interest
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