

Research Article

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Effects of nitrogen and phosphorus on *Microcystis aeruginosa* growth and microcystin production

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Abstract: In the present study, the effects of nitrogen (N) and phosphorus (P) on the growth of *Microcystis aeruginosa* and the production of microcystins (MCs) were investigated. The results showed that the growth of *M. aeruginosa* was not merely determined by N or P, but both nutrients were limiting for the species. Moreover, an excess of N and constant P in the culture medium could stimulate the growth of *M. aeruginosa*, whereas the growth of the species was inhibited in the culture medium containing excess of P and constant N. The optimal growth of *M. aeruginosa* was at an N:P ratio of 0.1 with the maximal optical density of 1.197 at 680 nm (OD_{680}), whereas the maximal microcystin-LR (MC-LR) content of $228.2 \mu\text{g}\cdot\text{L}^{-1}$ observed in the culture medium with an N:P ratio of 5. Interestingly, MC-LR production occurred under conditions of N starvation, thereby suggesting that the growth rate of *M. aeruginosa* was not related to MC-LR production under conditions of nutrient stress.

Keywords: *Microcystis aeruginosa*, nitrogen, phosphorus, eutrophication, nutrient conditions

1 Introduction

In recent years, the eutrophication of water bodies has become an increasingly serious problem [1]. Eutrophication can lead to frequent outbreaks of toxic cyanobacterial blooms. Such events are harmful to water bodies, aquatic ecosystems, and even human health [2] and are responsible for producing toxins, including microcystins

(MCs). MCs are widely distributed and commonly detected cyanotoxins produced by numerous toxic cyanobacteria, such as *Microcystis*, *Aphanizomenon*, *Planktothrix*, and *Anabaena* [3]. Of those, *Microcystis aeruginosa* has been reported as the main producer of MCs [4].

MCs are a group of cyclic heptapeptides and generally described as cyclo-(D-alanine-R1-DMeAsp-R2-Adda-D-glutamate-Mdha), which can induce toxicity by the inhibition of members of the protein phosphatase families protein phosphatases 1 (PP1) and protein phosphatases 2A (PP2A) [5]. To date, more than 90 derivatives of MCs have been identified [6]; among them, microcystin-LR (MC-LR), microcystin-RR (MC-RR), and microcystin-YR (MC-YR) are commonly found variants of MCs [7], and MC-LR is considered to be as the most frequently occurring toxin [8]. The guideline value of MC-LR suggested by The World Health Organization for safe drinking water was $1.0 \mu\text{g}\cdot\text{L}^{-1}$.

MCs are cyanotoxins and the secondary metabolites during cyanobacterial blooms, which would be released into the water as cyanobacterial cells lyse. Various methods for the removal of MCs have been reported in the literature from time to time, such as chlorine oxidation [9], ozonation [10], photocatalytic process [11], adsorption [12], and microbial degradation [13]. However, the photocatalytic process is likely to cause secondary pollution due to an unknown structure intermediate compound [14], adsorption is inadequate for the removal of dissolved MCs completely, ozonation and chlorine oxidation could generate harmful by-products into the ecosystem [15]. Therefore, the new efficient method for the removal of MCs merits investigation.

Studies have shown that various environmental factors such as pH, light, nutrients, temperature, and dissolved oxygen affect the production of MCs [16–18]. Phosphorus (P) availability is generally assumed to be an important limiter of cyanobacterial blooms and the production of MCs because some cyanobacteria can fix nitrogen (N) under N-limited conditions via N_2 fixation [19]. Conversely, the importance of N availability has also been shown in some studies [1,20]. Contradictory results for the impact of N:P ratios on cyanobacterial bloom and the production of MCs

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have also been widely reported [21,22]. The knowledge of how N and P interact to limit the cyanobacterial growth is essential in the eutrophication control and management [23]. Therefore, the objectives of this study are to investigate the effect of N availability, P availability, and N:P ratios on *M. aeruginosa* growth and the production of MCs. These results will be helpful to elucidate the influence of nutrients on cyanobacterial bloom and the production of MCs, which are essential in the MC removal and eutrophication control.

2 Materials and methods

2.1 Materials

All chemicals were of analytical grade and obtained from Xilong Chemical Co. Ltd., China. *M. aeruginosa* (FACHB-912) was obtained from Institute of Hydrobiology, Chinese Academy of Sciences, which was cultivated in 150 mL flask with 50 mL BG11 medium in an incubator with $22.5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of light power and 12:12 h (L:D) photoperiod at $25 \pm 1^\circ\text{C}$ [22]. Cells in the exponential phase were used as inoculum (approximately 7×10^6 cells per mL) in the following experiments.

2.2 Research methodology

To investigate the growth and MC production of the *M. aeruginosa* strain in relation to varying N and P concentrations, 1.0 mL culture of *M. aeruginosa* was added to 100 mL modified BG11 medium containing different concentrations (1.5, 0.75, 0.375, 0.15, and $0.075 \text{ g}\cdot\text{L}^{-1}$) of NaNO_3 and $0.04 \text{ g}\cdot\text{L}^{-1}$ $\text{KH}_2\text{PO}_4\cdot 3\text{H}_2\text{O}$ in a series of 250 mL flasks, the N:P ratios (mass units) were 100, 50, 25, 10, and 5, respectively. The flasks were placed in the incubator with the conditions of experiment as described above.

The modified BG11 medium contained H_3BO_3 $2.86 \text{ mg}\cdot\text{L}^{-1}$, $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$ $1.81 \text{ mg}\cdot\text{L}^{-1}$, $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$ $6 \text{ mg}\cdot\text{L}^{-1}$, $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ $75 \text{ mg}\cdot\text{L}^{-1}$, $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ $36 \text{ mg}\cdot\text{L}^{-1}$, ethylenediaminetetraacetic acid Na_2 $1 \text{ mg}\cdot\text{L}^{-1}$, and citric acid $6 \text{ mg}\cdot\text{L}^{-1}$.

For comparison, 1.0 mL culture of *M. aeruginosa* was added to 100 mL modified BG11 medium containing different concentrations (0.04, 0.2, 0.4, 1, 2, and $4 \text{ g}\cdot\text{L}^{-1}$) of $\text{KH}_2\text{PO}_4\cdot 3\text{H}_2\text{O}$ and $0.015 \text{ g}\cdot\text{L}^{-1}$ NaNO_3 in a series of 250 mL flasks, and the N:P ratios (mass units) were 1, 0.2, 0.1, 0.04, 0.02, and 0.01, respectively. The flasks were placed

in the incubator with the conditions of experiment as described above.

Two control tests were prepared as follows: 1.0 mL culture of *M. aeruginosa* was added to 100 mL modified BG11 medium containing different concentrations of NaNO_3 without $\text{KH}_2\text{PO}_4\cdot 3\text{H}_2\text{O}$, and the N concentrations were set as 30, 60, 120, 240, and $480 \text{ mg}\cdot\text{L}^{-1}$, respectively. A total of 1.0 mL culture of *M. aeruginosa* was added to 100 mL modified BG11 medium containing different concentrations of $\text{KH}_2\text{PO}_4\cdot 3\text{H}_2\text{O}$ without NaNO_3 , and the P concentrations were set as 2.7, 5.4, 10.8, 16.2, and $21.6 \text{ mg}\cdot\text{L}^{-1}$, respectively. Each treatment was performed in triplicate and incubated as previously described.

2.3 Analytical methods

Every 7 days, 1 mL of each culture was sampled under sterilized environmental conditions during the experimental period, optical density was measured at 680 nm, and the effects of N and P on the growth of *M. aeruginosa* were investigated. The algal density divided by time during the exponential growth phase was determined as specific growth rate, μ (day^{-1}) [24]. Total phosphorus (TP) was analyzed using ammonium molybdate spectrophotometric method (GB11893-1989, China), nitrate-nitrogen was determined by Ultraviolet spectrophotometry method (HJ/T 346-2007, the National Environment Protection Bureau of PR China). The utilization concentration of N (P) was calculated by subtracting the residual aqueous concentration of N (P) from the initial concentration of N (P).

For the measurement of MC-LR, 9 mL of the culture sample was freeze-dried at -20°C for storage until analysis, and then the freeze-dried sample was redissolved in deionized water, freeze-thawed three times, centrifuged at $4,000\times g$ for 30 min and passed through $0.45 \mu\text{m}$ fiber filter, the supernatant was applied to a solid phase extraction cartridge (Cleanert C18 500 mg per 6 mL cartridge, Phenomenex & Agela, Tianjin, China) [16], and the 1 mL collected elution after bath evaporation at $65\text{--}80^\circ\text{C}$ was analyzed using the high-performance liquid chromatograph (Ultimate 3000, Shenzhen rushing technology co. Ltd, China), which is equipped with a reverse C18 column ($5 \mu\text{m}$, $150 \text{ mm} \times 3.9 \text{ mm}$). The column temperature was 30°C , the mobile phase were methanol and 0.01 M ammonium acetate (55:45, v/v) at a flow rate of $1 \text{ mL}\cdot\text{min}^{-1}$, and the injection amount was $10 \mu\text{L}$. Statistical tests were carried out using Statistical Product and Service Solutions, 17.0, and at least three replicates of the samples were prepared.

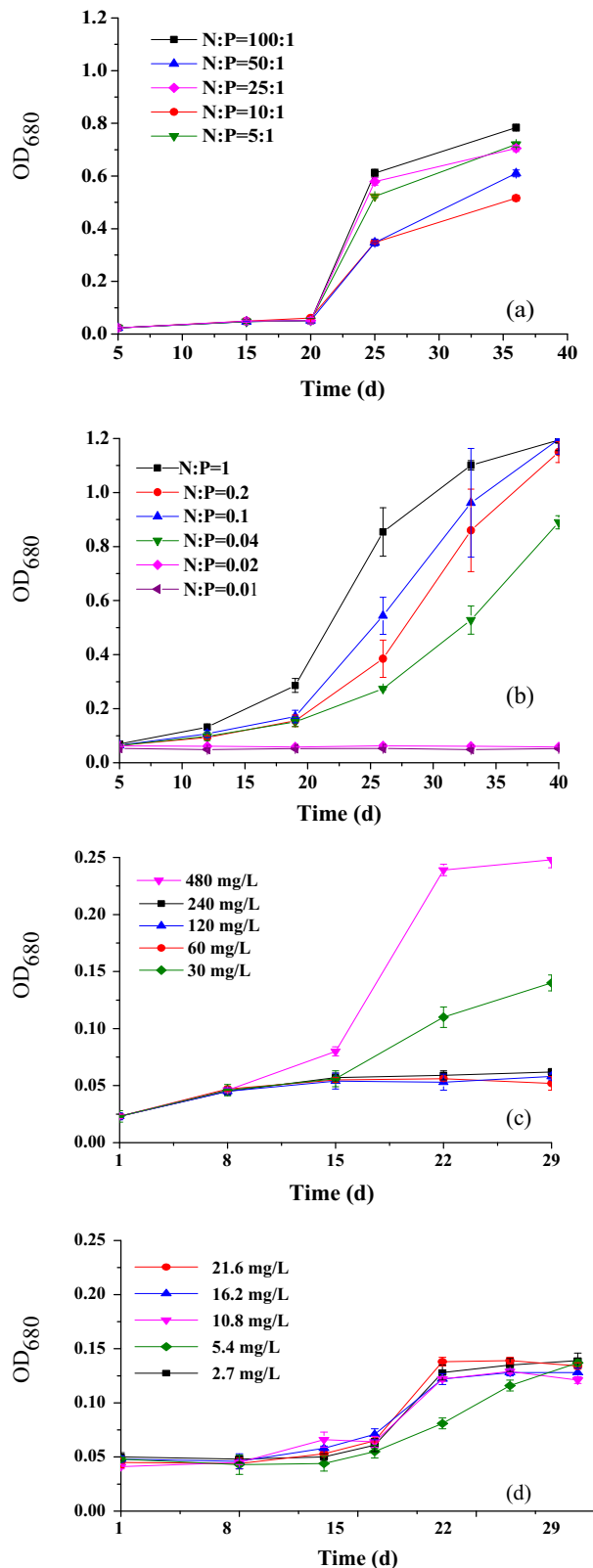


Figure 1: (a) The growth of *M. aeruginosa* cultured under constant P and varying N, (b) constant N and varying P, (c) different N concentrations without P, and (d) different P concentrations without N.

3 Results

3.1 Growth of *M. aeruginosa* under different nutrient conditions

When cultured under different N and constant P, no significant differences were observed in lag phase (Figure 1a); however, the algal densities increased differently in log phase, and the maximal densities of *M. aeruginosa* (OD₆₈₀) were 0.784, 0.61, 0.705, 0.516, and 0.72 at N:P supply ratios of 100, 50, 25, 10, and 5, respectively. Moreover, the specific growth rates were 0.035, 0.027, 0.031, 0.022, and 0.032 days⁻¹ at N:P supply ratios of 100, 50, 25, 10, and 5, respectively (Table 1). Therefore, the optimal growth of *M. aeruginosa* was at an N:P ratio of 100 with the maximal OD₆₈₀ of 0.784 and specific growth rate of 0.035 days⁻¹, whereas the growth of *M. aeruginosa* was inhibited at an N:P supply ratio of 10 with the OD₆₈₀ of 0.516 and specific growth rate of 0.022 days⁻¹.

When cultured under different P and constant N, the maximal OD₆₈₀ were 1.194, 1.15, 1.197, and 0.89 at N:P supply ratios of 1, 0.2, 0.1, and 0.04, respectively (Figure 1b). The specific growth rates were 0.038, 0.038, 0.039, and 0.028 days⁻¹ at N:P supply ratios of 1, 0.2, 0.1, and 0.04, respectively (Table 1). The growth of *M. aeruginosa* was inhibited at an N:P supply ratio of 0.04 with the OD₆₈₀ of 0.89 and specific growth rate of 0.028 days⁻¹. Moreover, the growth of *M. aeruginosa* virtually ceased at N:P supply ratios of 0.02 and 0.01, respectively, which means an excess of P would lead to growth limiting for *M. aeruginosa*. Therefore, the optimal growth of *M. aeruginosa* was at an N:P ratio of 0.1 with the maximal OD₆₈₀ of 1.197 and specific growth rate of 0.039 days⁻¹.

Increasing the N concentrations from 30 to 480 mg·L⁻¹ in P-free culture medium facilitated the varying of maximum OD₆₈₀ of *M. aeruginosa* from 0.052 to 0.248 (Figure 1c). Similarly, increasing the P concentration from 2.7 to 21.6 mg·L⁻¹ in N-free culture medium facilitated the varying of maximal OD₆₈₀ from 0.121 to 0.139 (Figure 1d). Both of them were far below the value of which cultured under different N:P supply ratios. A possible explanation for these findings was that the removal of P or N led to a starvation condition. The growth of *M. aeruginosa* occurred over a wide range of N and P supply ratios (Figure 1a and b), which means the growth of *M. aeruginosa* was not merely determined by N or P, but both N and P regulated *M. aeruginosa* growth.

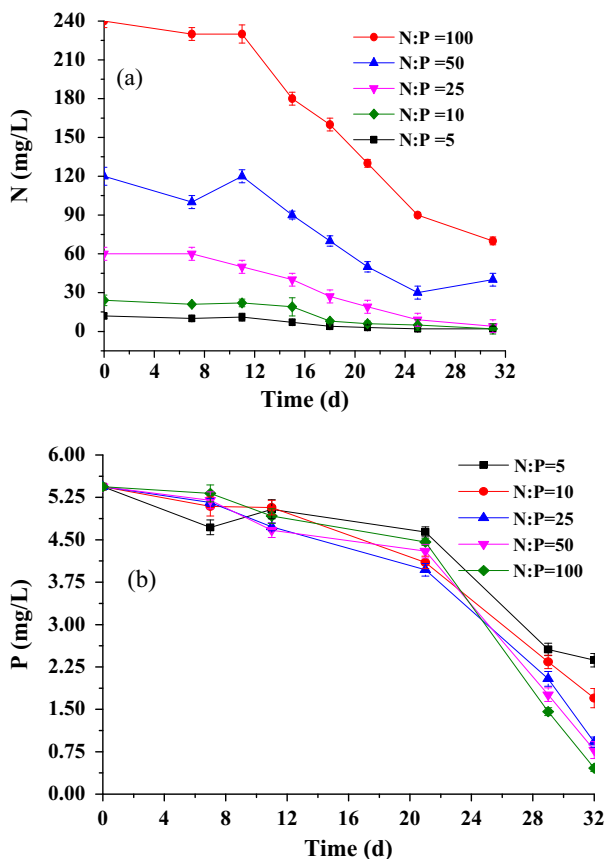
3.2 Variation of N and P concentrations in media

The variation of N concentrations in medium consisting of different N and constant P is shown in Figure 2a. It

Table 1: The specific growth rate of *M. aeruginosa* cultured under different N and P supply ratios

N:P	100:1	50:1	25:1	10:1	5:1	1	0.2	0.1	0.04	0.02 (0.01)
μ (day ⁻¹)	0.035	0.027	0.031	0.022	0.032	0.038	0.038	0.039	0.028	—

Notes: “—” Growth of *M. aeruginosa* ceased.

**Figure 2:** The variation of (a) N concentrations and (b) phosphate concentrations in medium consisting of different N:P ratios with time.

shows that the concentrations of N were declined at different N:P ratios with time, especially during 11–21 days. The utilization concentrations of N during the experimental period were 170, 90, 56, 22, and 10 mg·L⁻¹ with N:P supply ratios of 100, 50, 25, 10, and 5, respectively.

With respect to the variation of P concentrations, Figure 2b shows that the concentrations consumed of P during the experimental period were 4.98, 4.66, 4.52, 3.74, and 3.07 mg·L⁻¹ with N:P supply ratios of 100, 50, 25, 10, and 5, respectively. The consumed concentrations of P also increased with an increase in N:P supply ratios; however, Pearson's correlation coefficients between densities of *M. aeruginosa* and sig. values (two-tailed) indicated no significant relationship between *M. aeruginosa* growth and the concentrations consumed of P (Table 2), similar trend was noticed in the case of varying P and constant N (data not shown).

3.3 Relationship between growth of *M. aeruginosa* and MC-LR production

The relationship between MC-LR production and *M. aeruginosa* growth in medium consisting of different N and constant P is shown in Figure 3a. It shows that the contents of MC-LR were 128.2, 141.8, 199.6, 140.5, and 228.2 µg·L⁻¹ at N:P supply ratios of 100, 50, 25, 10, and 5, respectively, whereas the maximal densities of *M. aeruginosa* (OD₆₈₀) were 0.784, 0.61, 0.705, 0.516 and 0.72 at N:P supply ratios of 100, 50, 25, 10, and 5, respectively. Pearson's correlation coefficients between densities of *M. aeruginosa* and sig. values (two-tailed) indicated no significant relationship between *M. aeruginosa* growth and the production of MCs (Table 2).

Figure 3b shows that the contents of MC-LR were 51.9, 62.7, 79.2, and 51.3 µg·L⁻¹ at N:P supply ratios of 1, 0.2, 0.1, and 0.04, respectively, while the maximal densities of *M. aeruginosa* (OD₆₈₀) were 1.194, 1.15, 1.197, and 0.89 at N:P supply ratios of 1, 0.2, 0.1, and 0.04, respectively. Similarly, statistical tests indicated no significant

Table 2: Correlation between the algal density (OD₆₈₀) and the consumed concentrations of N (P), MC-LR production at varying N and constant P

OD ₆₈₀	The consumed N concentrations		The consumed P concentrations		MC-LR	
	Pearson corr.	Sig. (two-tailed)	Pearson corr.	Sig. (two-tailed)	Pearson corr.	Sig. (two-tailed)
	0.523	0.366	0.266	0.655	0.293	0.632

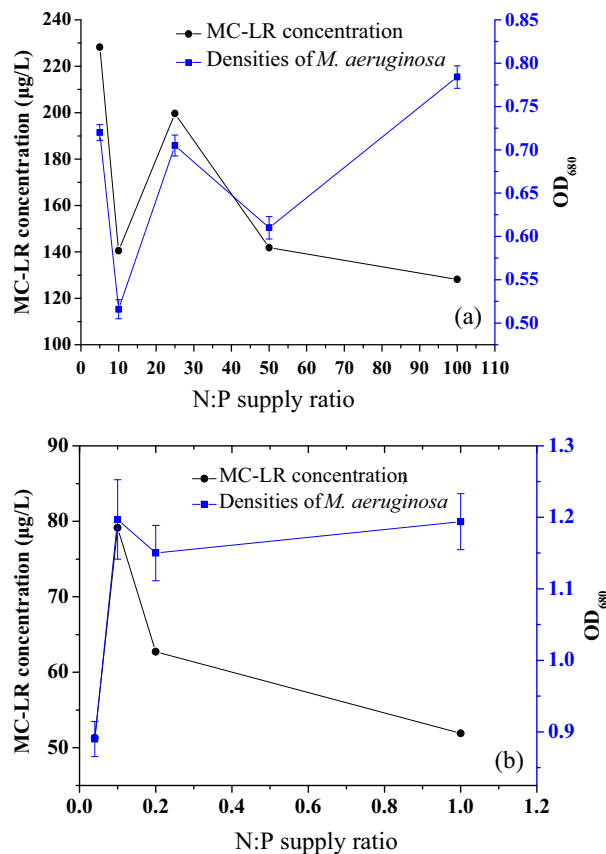


Figure 3: The variation of MC-LR concentration and densities of *M. aeruginosa* cultured under (a) different N and constant P and (b) constant N and varying P.

relationship between *M. aeruginosa* growth and the production of MCs (data not shown).

4 Discussion

Our results clearly demonstrated that the growth of *M. aeruginosa* was not merely determined by N or P, but both nutrients were limiting for the species. It is assumed that P is the key factor limiting *M. aeruginosa* growth and the production of MCs if the N:P ratio is over 20, whereas N is the limiting element if N:P is below 10 [25]; however, the present study demonstrated that *M. aeruginosa* growth and the production of MCs under different N:P supply ratios did not show a clear trend (Figures 1 and 3). Thus, the growth of *M. aeruginosa* was determined by the combination of N and P. Moreover, our study demonstrated that the growth of *M. aeruginosa* was enhanced at an N:P ratio of 100. The result was inconsistent with the study by Bortoli et al. [22], which reported that the lowest growth rate was N:P ratio of 100 in their experiment study. The possible explanation for inconsistent results

was the MC-LR content of our study was $128.2 \mu\text{g}\cdot\text{L}^{-1}$ in medium with an N:P ratio of 100, which was far lower than that of $228.2 \mu\text{g}\cdot\text{L}^{-1}$ in medium with an N:P ratio of 5 (Figure 3a); however, densities of *M. aeruginosa* in medium with an N:P ratio of 100 were higher than that of *M. aeruginosa* in medium with an N:P ratio of 5 (Figure 1), and the concentrations of consumed N indicated that more nitrate were absorbed under N-sufficient and P limitation conditions [26], which were mostly used for growth instead of storing in cell with an N:P ratio of 100, and it was assumed that N availability in cell would stimulate the synthesis of MCs [27]. These findings are similar to those from studies by Sevilla et al. [28], which reported that excess nitrate increased the *M. aeruginosa* PCC7806 growth rate without increasing the MC-LR production.

Our study also demonstrated that the growth rate and MC-LR production did not show a clear trend. The maximal growth rate occurred at an N:P ratio of 0.1 (Figure 1b), and the utilization P was not related to densities of *M. aeruginosa* increased (Table 2), which contradicted the previous finding by Wang et al. [29], which reported that *Microcystis* biomass was positively correlated with TP. Although the maximal MC-LR content appeared at an N:P ratio of 5 (Figure 3a), which differed from previous studies by Pimentel and Giani [30], which demonstrated that an increase in N concentration could stimulate the production of MCs, the possible explanation for these differences is that MCs are synthesized by different pathways [31].

Interestingly, the outcomes of MC-LR were also observed in N-starved conditions (Table 3), where the growth of *M. aeruginosa* cells was inhibited significantly (Figure 1d). Similar trends were found in a recent study, which reported N starvation of nutritionally replete cells could promote the biosynthesis of MC-LY [32]. The variation of *M. aeruginosa* growth with MC-LR content production under nutrient stress conditions should be deeply investigated in future, which will help improve the understanding of the role and function of MC.

5 Conclusion

This article studied the growth and MC-LR production of a *M. aeruginosa* strain in relation to varying N and P

Table 3: The variation of MC-LR concentration of *M. aeruginosa* cultured under varying P without N (N-starved conditions)

P concentration ($\text{mg}\cdot\text{L}^{-1}$)	2.7	5.4	10.8	16.8	21.6
MC-LR concentration ($\mu\text{g}\cdot\text{L}^{-1}$)	11.2	32.0	6.0	23.58	7.3

concentrations. The results indicated that the growth of *M. aeruginosa* was determined by the combination of N and P, and the optimum N:P ratios for the *M. aeruginosa* growth and MC-LR production were 0.1 and 5, respectively. The growth rate of *M. aeruginosa* was not related to MC-LR production, and the highest growth rate of *M. aeruginosa* did not produce the highest MC-LR concentration. These results hold potential applications toward understanding the influence of nutrients on *M. aeruginosa* growth and the production of MCs, and the choice of nutrient concentrations may be representative of realistic water conditions will be deeply investigated in future, which are important for controlling harmful algal blooms particularly *Microcystis* blooms.

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Author contributions: Benjun Zhou: writing – review and editing, visualization, and project administration and Zhen Wang: writing – original draft, and formal analysis.

Conflict of interest: Authors state no conflict of interest.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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