

Joint effects of five environmental factors on the growth of cyanobacterium *Microcystis aeruginosa*

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ABSTRACT

In many lakes and reservoirs, *Microcystis aeruginosa* is one of the dominant bloom species. Five environmental factors, including nutrients and physical factors, were selected to evaluate their effects and interactions on the growth of *M. aeruginosa* (FACHB-905) by joint analysis in a laboratory batch culture. The results indicated that all five factors affected the growth rate alone or in combination, and that their interactions were complex. This cyanobacterium strain preferred higher water temperature and alkaline conditions, while not requiring high illumination or high concentrations of nitrogen and phosphorus. Owing to these features the bloom of this cyanobacterium appears easily in nature. The form of nitrogen (nitrate or ammonium) also affected the assessment of *M. aeruginosa* bloom. The possibility of *M. aeruginosa* bloom would still exist even if the phosphorus concentration in the water column was very low. The result provided a good basis for the analysis and prediction of *M. aeruginosa* blooms in terms of environmental assessment, because the joint analysis of multiple factors would offer more valuable information than a univariate analysis.

Key words | environmental factor, harmful bloom, *Microcystis aeruginosa*, nitrogen, phosphorus, uniform design

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INTRODUCTION

Harmful algal blooms (HABs) in freshwater have become a hot topic across the world. They result in a deterioration in the quality of water resources, and also cause bad conditions that affect the growth and development of aquatic organisms in lakes or reservoirs (Kameyama *et al.* 2002; Backer *et al.* 2010). The formation mechanisms of HABs, as well as how to forecast or control the blooms, have therefore become a worldwide concern and a subject of serious debate (Tayaban *et al.* 2018). It is well known that an excess of green-blue algae (also usually termed cyanobacteria) in freshwater often causes occurrence of harmful blooms. The harmful bloom is considered to be the result of composite action by nutrients, light, temperature, etc. (Guildford & Hecky 2000; Kong & Gao 2005).

Since the occurrence and development of harmful blooms depend on diverse environmental factors, many studies focusing on environmental effects on algal proliferation have been carried out. Environmental factors evaluated in previous studies include nitrogen, phosphorus, carbon, heavy metals, illumination, water temperature, pH, etc. (Fujii *et al.* 2010; Hagstrom *et al.* 2010). Although certain basic rules for HABs were ascertained from previous reports, many studies evaluated only one or two factors. Sometimes results were inconsistent with others. In contrast only a few studies on *Microcystis aeruginosa* have considered multiple factors. Yuan *et al.* (2008) studied the effects of three factors on the proliferation of *M. aeruginosa* with an orthogonal design experiment. Their results gave us more information than some univariate experiments. However, the limitations of orthogonal experiments, which could not include many factors or their levels, meant that few factors were included in

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the analysis. Studies using many experimental treatments would incur too much expenditure and workload, and those using fewer factors or levels only offer a limited explanation of blooms. Quiblier *et al.* (2008) also conducted multi-factor studies in batch culture. They used natural water and added some nutrients; their main subject was the phytoplankton community. It has thus been shown that more studies including more factors are essential to bloom analysis.

The Uniform Design method (UD) was devised by Professors Fang and Wang in 1978, and is an important method for application in virtual experimental and solidity designs; UD has become a standard tool in experimental design over the last two decades (Fang & Ma 2001; Winker & Lin 2011). Compared with other statistical methods, UD reduces the number of experiments in a multiple-dimension optimization and allows the largest possible number of levels for each factor (Wu *et al.* 2013). It has been used successfully in many experiments of condition optimization (Peng *et al.* 2014). Compared with the common orthogonal design method, this method has some particular advantages. First, only one experiment is needed for each level of each factor, and the experimental counts are equal to the level counts; the lower number of experimental treatments will reduce cost and workload significantly. Secondly, it is both convenient for analyzing interactions among experimental factors and helpful in developing a mathematical model. Owing to these advantages, UD has hitherto been successfully applied in many research areas (Liang *et al.* 2001; Mehri & Ghazaghi 2014).

Five environmental factors showing effects on the growth of *M. aeruginosa* were chosen to do a basic integrative study. The five factors were nitrogen, phosphorus, temperature, pH and illumination. The joint effect of these factors on the growth of *M. aeruginosa* was the main aim of this study, but we also try to test the different impacts between ammonium and nitrate. We hope that the optimal conditions obtained from this study will be helpful in understanding the complexity of cyanobacterial blooms.

MATERIAL AND METHODS

Cyanobacterium strain and culture conditions

The cyanobacterium strain *Microcystis aeruginosa* (FACHB-905) was bought from the Freshwater Algae

Culture Collection of the Institute of Hydrobiology, CAS (<http://algae.ihb.ac.cn>). The cyanobacterium seed was cultured in BG-11 medium in the culture box prior to the experiments. Culture conditions for the seed were as follows: water temperature 25 ± 0.5 °C; pH 7.5–8.5; illumination $28.5\text{--}34.2 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$; and photoperiod 14 h:10 h (light:dark).

Experimental materials, equipment and environments were sterilized or disinfected before the experiments. *M. aeruginosa* was cultured under axenic conditions. All operations and counting processes were performed under aseptic laboratory conditions.

Experimental designs

The five environmental factors selected for the study were nitrogen (nitrate and ammonium in different experiments), K_2HPO_4 , illumination, water temperature, and pH. The optimal table of the Uniform Design ($U^*_{12} 6^2 \times 3^2 \times 4$), designed using Data Processing System (DPS) software (Tang & Feng 2007; Kong *et al.* 2012), was used for this study. The levels of the five factors and their arrangements are presented in Table 1. Twelve treatments were set for each experiment, as well as three repetitions for each treatment.

Table 1 | Experimental levels of each factor and their arrangements in the Uniform Design experiments (1 klx = $11.4 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$)

Treatment number	Nitrogen (mmol L ⁻¹)	Phosphorus (mmol L ⁻¹)	Illumination (klx)	Temperature (°C)	pH
C01	4.0	0.05	1	15	7
C02	0.1	0.4	3	35	7
C03	0.01	0.2	1	25	5
C04	1.5	0.1	1	35	11
C05	2.5	0.01	3	35	5
C06	0.5	0.1	5	15	5
C07	0.1	0.01	3	15	11
C08	1.5	0	5	25	7
C09	4.0	0.2	5	25	11
C10	2.5	0.4	3	15	9
C11	0.5	0	1	25	9
C12	0.01	0.05	5	35	9

The batch culture method was used for both experiments, i.e. no nutrients were added during the experiments.

- (1) An appropriate amount of *M. aeruginosa* in its exponential growth stage was transferred into a 5 L triangular flask and all the components were added to the BG-11 culture medium except nitrogen and phosphorus. After being shaken gently, 100 mL of mixed liquid with well distributed *M. aeruginosa* was pipetted into a 250 mL triangular flask, which was marked as one repetition of an experimental treatment.
- (2) Nitrate and K_2HPO_4 were added to each treatment, according to the concentrations presented in Table 1. Three repetitions were specified for each treatment.
- (3) After controlling the pH of each treatment, the flasks were placed under their temperature and lighting conditions, as listed in Table 1. For the second experiment, the above steps were repeated, but using ammonium instead of nitrate.

Data collection and analysis

The densities of *M. aeruginosa* were measured microscopically in the blood-cell-counting chamber every two days. These flasks were shaken twice a day during the experiments. The growth rates (μ) of this cyanobacterium were calculated using the formula:

$$\mu = (\ln N - \ln N_0)/t \quad (1)$$

where t indicated the days of exponential growth period, N was the density of the t day, and N_0 was the initial density at the beginning of the exponential growth period. DPS software (Tang & Feng 2007) was used for the subsequent statistical analysis and data fitting. The software OriginPro was used to plot the figures.

RESULTS

Brief introduction to the growth of *M. aeruginosa*

In both experiments minor increases and lower final densities were detected for most treatments. *M. aeruginosa* increased greatly and got the highest final densities under

the conditions of treatment C04 in both experiments. In the ammonium experiment the treatment C04 represented the best growth of *M. aeruginosa*, outshining other treatments, in which the growth curve went up rapidly. Among the other treatments, C02 showed a better growth condition, but its cell density was close to that in treatments C08, C11 and C12. In the nitrate experiment the cell densities increased sharply in both treatment C04 and treatment C09, although C04 showed a little higher density. Some treatments showed minor increases, and others had nearly no increase. The final densities with the two nitrogen forms were quite different; the difference between the maximum and minimum was huge (Figure 1).

Comparing the final densities in terms of the treatment, the variation trends between the two nitrogen experiments were quite similar. Most treatments in both experiments, such as C01, C02, C06, C07, C10, C11 and C12, had similar final densities, while the contrast in treatment C09 showed a quite different variation, where the density in the nitrate experiment was ninety times higher than that in the ammonium experiment. The final densities of C03 and C05 were higher in the ammonium experiment than in the nitrate experiment, although all their amounts were low.

In both nitrate and ammonium experiments, *M. aeruginosa* had the best increase of density under treatment C04, but little growth in treatment C06. This formed a sharp contrast of proliferation (Figure 2). The treatment C04 provided lower concentrations of nitrogen and phosphorus, and lower illuminance, but higher temperature and pH; the treatment C06 provided almost the opposite growth conditions, except that the nitrogen concentration decreased more (Table 1). These differences clearly indicated the favorite growth condition of *M. aeruginosa*. This cyanobacterium preferred higher water temperatures and an alkaline environment, but did not require higher illumination or higher concentrations of nutrients (nitrogen and phosphorus). Phosphorus was not added in the treatments C08 and C11 in this study; however, the cyanobacteria in them kept growing to some extent (Figure 2). The growth rates of C09 showed great difference in terms of nitrogen forms, which was consistent with the analysis of final density.

The effect of temperature was also obvious in this study. The growth rates in the high-temperature group (e.g. C02, C04, C05 and C12) were higher than those of the low-temperature group

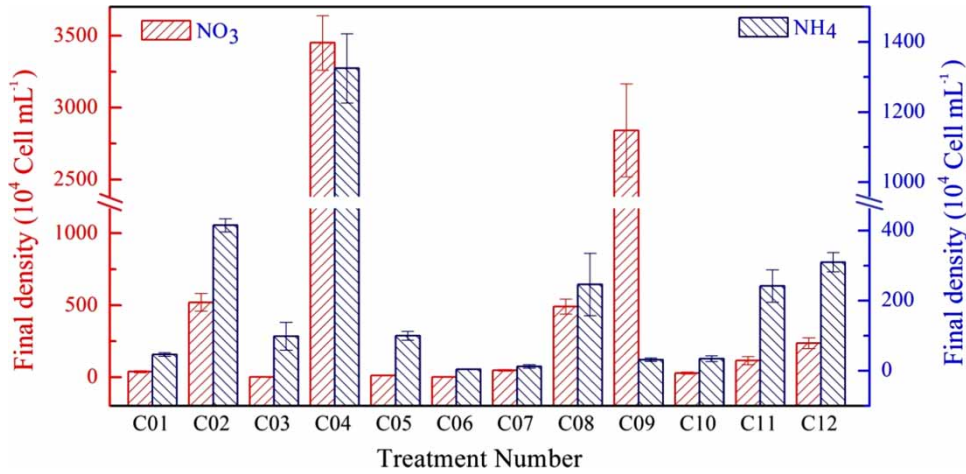


Figure 1 | Final densities of *M. aeruginosa* in both experiments using ammonium or nitrate as nitrogen resource.

(e.g. C01, C06, C07 and C10). Low temperature was an important limitation for the bloom (Figure 2).

Regression equations

The stepwise regression of a quadratic multinomial to the growth rate was performed for both experiments, in order to derive optimal growth conditions and understand

the relationships among these factors. The regression equation in the nitrate experiment was as follows ($R = 1$, Durbin-Watson value $d = 1.6$):

$$Y_1 = -0.500 + 0.003[T] - 0.043[N]^2 + 7.15[P]^2 - 0.005[L]^2 - 0.366[N][P] - 0.020[N][L] + 0.04[N][pH] - 0.348[P][pH] + 0.010[L][pH] + 0.001[T][pH]$$

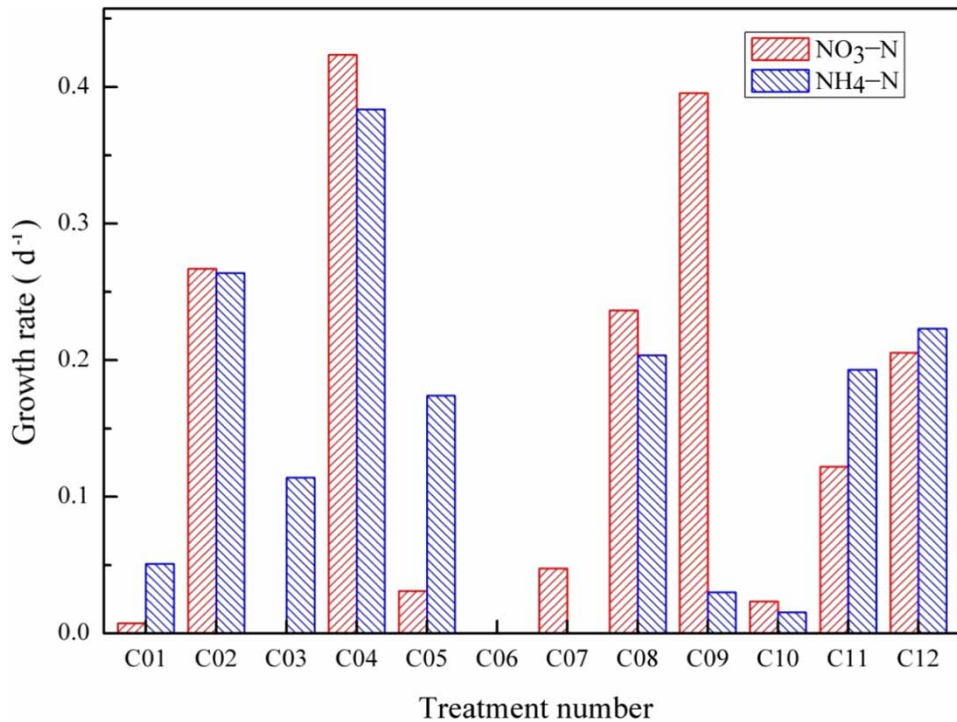


Figure 2 | Comparison of the growth rates between nitrate and ammonium among the 12 treatments.

where [N], [P], [L], [T], and [pH] stood for the concentrations or levels of NO₃-N, HPO₄-P, illumination, water temperature, and pH value, respectively. The coefficients of *R* and *d* suggested that the model was efficient and could explain most of the experimental data. The optimal growth conditions calculated from the equation were as follows: NO₃-N, 3.86 mmol L⁻¹; HPO₄-P, ~0 mmol L⁻¹; illumination, 2.86 klx (32.6 μmol m⁻² s⁻¹); water temperature, 30.56 °C; and Ph, 10.73. This gave a maximum predicted result for the growth rate as 0.981. Obviously, the zero phosphorus concentration in this equation was a calculated result, and did not mean this cyanobacterium did not need phosphorus nutrient. It meant that lower phosphorus concentrations could satisfy its growth.

With regard to the ammonium experiment, the regression equation was as follows (*R* = 1.00, Durbin-Watson value *d* = 2.47):

$$Y = -1.838 + 1.042[P] + 0.028[T] + 0.344[pH] + 0.013[L]^2 - 0.019[pH]^2 + 0.001[N][T] - 0.410[P][L] + 0.007[P][T] - 0.003[L][T] - 0.001[L][pH]$$

The symbols in this equation are the same as in the previous equation except that [N] stood for ammonium (NH₄-N) concentration. The coefficients of *R* and *d* also suggested that this model was efficient. The optimal growth conditions calculated from the equation were as follows: NH₄-N, 0.89 mmol L⁻¹, HPO₄-P, 0.40 mmol L⁻¹; illumination, 11.4 μmol m⁻² s⁻¹; water temperature, 34.99 °C; and pH, 8.65. This gave the maximum predicted result for the growth rate as 0.977.

This regression equation also showed that independent or joint impacts of environmental factors on the growth of *M. aeruginosa*, as well as complex interactions, still existed. The interactions were a little different from the result of nitrate experiment. Lower ammonium concentration was found to be propitious for the proliferation of *M. aeruginosa*, which was consistent with the description presented in the previous section of this paper.

Verification experiments

Some verification experiments were carried out in order to validate the accuracy of the two regression equations; some levels were set for these factors at random. The results

Table 2 | Pearson correlations of the simulated results and experimental results for the fitting equations of nitrate (NO₃-N) and ammonium (NH₄-N) (at 0.05 level, two-tailed test)

Nitrogen form	Growth rate		Pearson correlation	
	Simulation	Experiment	Coefficient	Significance
NO ₃ -N	0.819	0.601	0.881*	0.048
	0.079	0.409		
	0.002	0.005		
	0.262	0.424		
	0.109	0.205		
NH ₄ -N	0.975	0.772	0.889*	0.043
	0.098	0.016		
	0.317	0.437		
	0.664	0.384		
	0.416	0.223		

*Denotes the significant correlation at 0.05 level, two-tailed test.

of Pearson correlation between the simulated results from the equations and experimental results are listed in Table 2 (the full table is shown in the Supplementary Material, available with the online version of this paper).

In nitrate experiments, the correlation analysis showed a significant result: the coefficient of Pearson correlation reached 0.881 (*p* < 0.05). The correlation in ammonia experiments was also significant, with a coefficient of 0.889 (*p* < 0.05). These results indicate that, within the experimental range, the two fitting equations could better represent the link between the *M. aeruginosa* growth and the five environmental factors.

DISCUSSION

Optimal growth conditions of *M. aeruginosa*

The optimal values indicated that this cyanobacterium preferred higher water temperatures and an alkaline environment, but did not need higher illumination or higher concentrations of nitrogen and phosphorus. That is to say, this cyanobacterium strain had highly adaptive abilities in the environment. This result was consistent with some reports about cyanobacteria (Dokulil & Teubner 2000). Cyanobacteria proliferations have been reported in oligotrophic and mesotrophic freshwater bodies (Jacquet *et al.* 2005). A strong *M. aeruginosa* proliferation was observed in the Djoudj pond with higher nitrogen concentrations,

whereas soluble reactive phosphorus (SRP) concentrations were always low (Berger *et al.* 2005; Quiblier *et al.* 2008). The results of this study were quite similar to the field observations. In addition, the designed N/P (nitrogen/phosphorus) ratio in treatment C04 was 15:1, which was also the same as the Redfield ratio and similar to other reports (Yi *et al.* 2005; Zhang & Hu 2011). This ratio was also a good condition for the increase of microalgae.

This regression equations showed that all the five environmental factors had independent and/or joint impact on the growth of *M. aeruginosa*. The interactions were complex and existed between chemical and physical factors, which could not be indicated by univariate result. So the joint result of multiple factors was more necessary for bloom study.

Generally speaking, the difference between the univariate and multivariate results was not big (Zhang *et al.* 2011a, 2011b, 2011c), but the multivariate result would be more suitable for bloom assessment. Also, the fitting model was useful to forecast cyanobacterium growth under various conditions. In addition, the bloom may be limited by some factors that were not tested in our study, so further studies including more key factors should be conducted in laboratory and in field study. It is a good basis for bloom analysis.

Influence of nitrogen forms

The results of this study suggested not only the influence of environmental factors on the growth of *M. aeruginosa*, but also reflected the different effects caused by the nitrogen forms. Because, as mentioned before, the difference between the two experiments was just the nitrogen forms, while the optimal conditions and their interactions were not the same (Figure 2 and fitting equations). Lin (1988) reported that although micro-algae used ammonia nitrogen during the summer, nitrate nitrogen was more important during the spring. So it was necessary to monitor and analyze nitrogen forms for bloom study. The changing of nitrogen forms also affected the adaptation of *M. aeruginosa* to other environmental conditions. For example, the growth in treatment C09, which had the highest nitrogen concentration in the two experiments, varied greatly. It was consistent with some reports. Some results of previous studies indicated that some algae preferred ammonium, but high ammonium concentration inhibited the growth of micro algae; however

high nitrate concentrations would not show toxicity to algae growth (Kameyama *et al.* 2002; Zhang & Hu 2011).

The nitrogen forms in the aquatic ecosystem were changeable; the transformation between nitrogen forms occurred frequently owing to biological processes. A combined analysis including various nitrogen forms in the water column would be useful for phytoplankton study.

About phosphorus storage

Obviously, the zero phosphorus concentration in this study was a calculated result, which did not mean this cyanobacterium did not need phosphorus nutrient (Wang *et al.* 2018). It meant that lower phosphorus concentrations could satisfy its growth. It was consistent with the previous analysis of phosphorus storage in this paper. On the other hand, Quiblier *et al.* (2008) found that SRP alone had no effect on phytoplankton biomass. However, when SRP was added simultaneously with dissolved inorganic nitrogen (DIN), the biomass increase was greater than when DIN was added alone. Their experiment also indicated that phosphorus was not non-essential for phytoplankton growth, but that its impact was affected by other factors.

Some literature reported that some microalgae had the ability to store phosphorus (Li *et al.* 2001; Zhang *et al.* 2006). The phosphorus was not added into the treatments C08 and C11 in this study. However, the cyanobacteria in them kept growing to some extent (Figure 2). This suggested that the cyanobacterium could store phosphorus. Details of the means by which *M. aeruginosa* is able to store phosphorus should be examined in further research, as it is useful for bloom study. It would be not adequate if only the phosphorus concentration in a water column was monitored; even if the phosphorus concentration was low in a water column, a bloom was still possible. It may also affect the analysis of the N/P ratio.

Enlightenments gained from the physical factors

The current results suggest that water temperature had a strong influence on the growth of *M. aeruginosa*. This was consistent with monitoring results *in situ*, with many cyanobacteria blooms occurring when water temperature increased during the summer months (Zheng *et al.* 2008).

The data of Berger *et al.* (2006) suggested that cyanobacteria were regulated annually in a lake as a result of changes in water temperature. The temperature could indeed have a major impact on the development equilibrium between cyanobacterium and diatom. The higher water temperatures (between 27 and 31 °C from May to October) favored the cyanobacteria, whereas diatoms appeared to be more competitive when temperatures were at their lowest (between 20 and 24 °C from November to February) (Quiblier *et al.* 2008). The optimal values of temperature were a little higher than the reports of *Cylindrospermopsis raciborskii*, whose optimum temperature was around 25 to 30 °C (Saker & Griffiths 2000; Briand *et al.* 2004); this difference may affect the competition between the cyanobacteria.

This cyanobacterium strain did not require strong light for growth. These features would increase the possibility of an *M. aeruginosa* bloom, and the shading method for limiting algal growth may be risky for controlling *M. aeruginosa* blooms (Zhang *et al.* 2011c): the self-shading of high-density blooms was easy to weaken to some degree. Quiblier *et al.* (2008) suggested that self-shading could be disrupted by the stirring effect of winds or water flow, thus boosting *M. aeruginosa* growth. So the generally accepted view that cyanobacteria development was favored by water stability was challenged.

The pH value showed some independent effect on and/or interaction with cyanobacterium growth. The pH did not show an independent effect in nitrate experiments, but it showed significant interactions with the other four factors. Ma *et al.* (2005) reported that the effect of pH changed with the phosphorus concentration in a competitive experiment between cyanobacteria and green algae, and their finding was consistent with ours. Li *et al.* (1996) hypothesized that the pH factor could correlate with carbon in the culture system of *Spirulina platensis*. Some people had considered that pH was a bloom result rather than an impacting factor, while this experiment indicated that pH could not be ignored in a bloom study.

CONCLUSIONS

Microcystis aeruginosa (FACHB-905) preferred a higher water temperature and a more alkaline environment, but

did not require higher illumination or higher concentrations of nitrogen and phosphorus. These conditions are relatively easy to meet in the natural environment, so blooms of *M. aeruginosa* often appear in freshwater. Five environmental factors in this study produced independent or joint impacts on the growth of *M. aeruginosa*, as well as their interactions being quite complex. It is most necessary to carry out a comprehensive analysis with multivariate experiments. This information would be quite beneficial in the analyzing and forecasting of cyanobacteria blooms.

Nitrogen has forms in freshwater usually; the forms of nitrogen existing are an important subject for bloom analysis. The designed difference between the two experiments in this study was just the nitrogen form, and their results, such as the fitting equations, the optimum growth conditions and their interactions, were not quite the same. When studying blooms, we should pay attention to both the nitrogen concentration and its forms in the water column. The combined effect of different forms of nitrogen on phytoplankton growth should also be studied in future.

The experimental results showed that *M. aeruginosa* could show good proliferation under conditions of lower phosphorus concentration; it could still grow in the two treatments whose phosphorus concentration was 0 mmol L⁻¹. It indicated that this cyanobacterium had a good ability to store phosphorus to some extent. Then, lower phosphorus concentrations in water column do not mean the non-occurrence of blooms. A bloom may still appear if the cyanobacteria have some bodily stored phosphorus. It is preferable to monitor the nutrient concentration in a water column continually for a long period, in order to know the variation of nutrients.

This primary study is a good basis for bloom analysis; experiments including more environmental factors will give us a better understanding of bloom occurrence.

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