



Synergy Effect of Environmental Factors on the Growth and Toxins Production by *Microcystis aeruginosa*

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ABSTRACT

Microcystis is one of the most common algal genera that causes water eutrophication. The effect of water quality on the growth and toxins production of *Microcystis* has been attracting high research attentions. The relationship of different combinations of temperature, illumination intensity, nitrogen and phosphorus with the growth and toxin production of *Microcystis aeruginosa* was explored through an orthogonal test. Results showed that illumination intensity influences the growth of *Microcystis aeruginosa* mostly, followed by temperature, total nitrogen (TN) and total phosphorus (TP). Illumination intensity affects the toxin production of *Microcystis aeruginosa* most significantly, followed by TP, TN and temperature successively. 200Lx illumination intensity, 22°C temperature, 20 mg.L⁻¹ TN and 0.6 mg.L⁻¹ are the optimum toxin production conditions for *Microcystis aeruginosa*.

INTRODUCTION

Harmful cyanobacterial blooms are one of the most severe problems in freshwater ecosystems. A Survey of the United Nations Environment Programme (UNEP) reported that 30-40% of lakes and reservoirs in the world are suffering from eutrophication (Ingrid & Jamie 1999). *Microcystis aeruginosa* is the most successful bloom-forming organisms in many freshwater ecosystems (Lin et al. 2019). *Microcystis* is not only the dominant algae in eutrophic water but also toxin-producing algae that can produce Microcystins (MCs) (Puschner et al. 1999, Azevedo et al. 2002). These toxins are harmful to a wide range of organisms (Gilbert 1996, Liu et al. 2006, Tilimanns et al. 2008). Microcystins are a kind of heptad peptide monocyclic compound. Its ring structure is D-Ala-L-X-red-β-methyl-D-isoaspartate-L-Z-Adda-D-isoglutamic acid-N-methyl dehydroalanine. Currently, 75 isomers of MC have been discovered (Van Apeldoorn et al. 2006). MCs are water-soluble and have strong heat resistance, but are difficult to be deposited or adsorbed. In 1998, the World Health Organization (WHO) has determined the base value of MCs in water at 1 µg/L (WHO 1998).

Both existing Chinese and foreign researches on toxin production mechanism of *Microcystis* mainly focus on genetic and environmental factors (Joung et al. 2011, Jähnichen et al. 2011, Reichwaldt & Ghadouani 2012). Environmental factors mainly include water temperature, illumination

intensity and nutritive salts like nitrogen and phosphorus (Watanabe & Oishi 1985, Rapala 1988, Oh et al. 2000, Kameyama et al. 2002). However, no agreement on the influence of the environment on toxin production mechanism has been reached yet. Utkilen & Gjølme (1992) pointed out that illumination may be the only influencing factor of toxin production of *Microcystis*. Wiedner et al. (2003) cultured *Microcystis* PCC7806 continuously, finding that the MC content per unit cell has a significant positive correlation with illumination intensity under limited illumination, but turns into a negative one under adequate illumination. Kameyama et al. (2003) conducted batch cultivation of *Microcystis viridis* NIES102 under different concentrations of nitrogen and phosphorus but didn't find a significant relationship between intracellular MC and nitrogen concentration within the logarithmic growth period. Downing et al. (2005) studied *Microcystis* PCC7806 and *Microcystis* UV027, and found that MC concentration per unit cell is related to N/P. Although some studies have involved the effect of illumination, nitrogen and phosphorus content on toxin production mechanism of *Microcystis*, growth and toxin production of algae have abundant influencing factors and no research has explored the effect degrees of different factors on toxin production mechanism of *Microcystis* and their combined effect. Hence, it is necessary to study the combined effect of different factors on the growth and toxin production characteristics of algae.

Based on the effect of a single factor on the growth and toxin production of *Microcystis*, this paper further explored the combined effect of nitrogen, phosphorus, temperature and illumination by using the L9(3⁴) orthogonal test.

MATERIALS AND METHODS

Experimental Chemicals

Methanol: chromatographically pure ($\geq 99.9\%$), Sinopharm Chemical Reagent Co., Ltd. Trifluoroacetic acid (TFA): chromatographically pure ($\geq 99.5\%$), Sinopharm Chemical Reagent Co., Ltd. Algal toxin-LR standards: 50 μg , Biobase Co., Ltd.

Experimental Methods

- Algae culture:** Testing *Microcystis* (*Microcystis aeruginosa* FACHB-315) was bought from Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB-collection) and then cultured in an HP 1000GS incubator under artificial climate. The culture medium was improved BG-11. Culture conditions are: 30°C, 1000-1500 Lux illumination intensity and 14:10 illumination/dark. The position of the triangular flask was adjusted randomly and shook 2-3 times regularly every day during the culture.
- Solution preparation:** Monopotassium phosphate solution: 6.8 g monopotassium phosphate was dissolved in 1,000 mL ultrapure water.
Phosphate buffered solution: pH of the monopotassium phosphate solution was adjusted to 3.0 by 20% phosphoric acid solutions.
Leaching solutions: 10 mL ultrapure water and 10 mL 20% methanol aqueous solutions.
Elution solution: 0.1 mL TFA (chromatographically pure) was dissolved into 100 mL methanol (chromatographically pure).
- Test conditions setting:** After the *Microcystis* had been starved for 3 days, concentrations of nitrogen and phosphorus in the culture medium were adjusted by $\text{Ca}(\text{NO}_3)_2$ and K_2HPO_4 . Culture temperature, illumination intensity and illumination/dark of *Microcystis* were set by the illumination incubator. The experiments were designed based on the orthogonal test (Table 1).
- Algae cell density measurement:** Blood counting chamber method: water sample was stirred evenly. Three test samples were collected from the upper, middle and bottom layer of the triangular flask respectively to measure the number of algae cells. The mean of three measurements was taken as the final results.

Table 1: Orthogonal experimental scheme.

Number	Temperature (°C)	Light intensity (Lx)	TN (mg/L)	TP (mg/L)
1#	22	200	20	0.4
2#	22	500	60	0.6
3#	22	800	100	1.0
4#	25	200	60	1.0
5#	25	500	100	0.4
6#	25	800	20	0.6
7#	28	200	100	0.6
8#	28	500	20	1.0
9#	28	800	60	0.4

The specific growth rate of *Microcystis* during the logarithmic phase was analyzed according to its growth curves under different culture conditions (Margarita et al. 2002).

$$K = \frac{\ln N_t - \ln N_0}{t - t_0} \quad \dots(1)$$

Where K is the relative growth rate of cells, t_0 means the initial time of the logarithmic phase, N_0 is the initial concentration of the logarithmic phase, and N_t is the algae concentration at time t.

- Absorbance value of *Microcystis*:** Before the test, some *Microcystis* solution at stable phase was taken and scanned by ultraviolet spectrophotometer under 200-800 nm wavelength, determining that 600-700 nm is the optimum absorption wavelength. The absorbance value of *Microcystis* solution under 600-700 nm was tested every other day.
- Determination of MCs:** 80 mL *Microcystis* solution was taken and extracted by microwave digestion/extraction instrument (Italy MILESTONE) for 10 min under 100°C. The extract was then filtered by 0.45 μm fibre filter membrane to collect *Microcystis* solution.

Activation of C18 solid-phase extraction column (Bona SPE500mg/3cc): 10mL methanol was injected into the extraction column activated by 10mL water when the methyl alcohol level reached the upper screen of the extraction column.

The collected *Microcystis* solution was injected into the activated solid-phase column for enrichment. The effluent was reinjected into the column again for secondary enrichment.

After sample enrichment, the C18 solid-phase extraction column was rinsed by the leaching solution (10 mL pure water and 10 mL 20% methanol solution).

MCs in the column was eluted with 10 mL elution solution. The effluent was collected.

The effluent was decompressed and steamed to dry by a rotary evaporator (Shanghai HongjiRe-52cs). Add 0.5 mL 50% methanol solution until the effluent was completely dissolved. The 2 mL effluent was used to test.

Detection conditions of HPLC (Varian LC-210): mobile phase: methanol (V): phosphate buffered solution (V)= 57:43; flow rate: 1 mL/min; detection wavelength: 238 nm. 20 uL sample was injected. Three parallel tests were conducted.

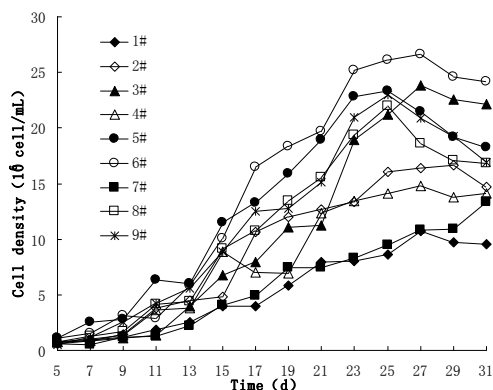
After finishing the test, the HPLC was rinsed by 10% methanol aqueous solution firstly to eliminate salts and then washed with pure methanol. The experimental design, data acquisition and data processing process shall comply with QA/QC requirements.

RESULTS AND DISCUSSION

Growth Curve of *Microcystis*

Growth curves of *Microcystis* under different culture conditions in the orthogonal test are shown in Fig. 1. *Microcystis* in 1#, 2#, 3#, 4# and 7# begin the logarithmic growth on the 9th day and the growth enters into the stable phase on the 27th day. The growth of *Microcystis* in 5# and 6# enters into the logarithmic phase on the 5th day. *Microcystis* in 5# and 6# grows quickly and the number of algae cells reaches the peak on the 23rd day. *Microcystis* in 8# and 9# begin to grow logarithmically on the 7th day and then the stable phase on the 25th day. Meanwhile, *Microcystis* in 1#, 4# and 7# under 200Lx illumination intensity grow slowly with the low algae biomass. This indicates that low illumination intensity can inhibit the growth of *Microcystis* significantly.

The specific growth rate and the maximum biomass of *Microcystis* at the logarithmic phase were further analyzed



(1-9 # are corresponding to those in Table 2)

Fig. 1: Variation curves of *Microcystis* density under different culture conditions.

Table 2: Comparison of growth rate and the maximum biomass of *Microcystis* under different culture conditions.

Number	Compared growth rates during logarithmic growth /d	Maximum biomass (10^6 a/mL)
1#	0.123	10.75
2#	0.137	16.65
3#	0.170	23.8
4#	0.133	14.8
5#	0.167	23.35
6#	0.176	26.6
7#	0.124	13.375
8#	0.158	21.975
9#	0.164	22.95

according to the growth curves under different culture conditions (Table 2). There is the largest biomass (26.6×10^6 /mL) in 6#, which is followed by 3# (23.8×10^6 /mL). The growth rates of *Microcystis* in 6# and 3# are 0.176 and 0.170, respectively. The best growth of *Microcystis* was in 6# in these treatments which might be from the compensation of different factors because it is supplied with lower nitrogen content and the longest illumination. The lowest maximum biomass (10.75) and growth rate (0.123) were in 1# due to the lowest temperature, nutrient salt content and short illumination. Moreover, there is a positive correlation between the maximum biomass and the growth rate in these treatments.

Variation of MCs

Effect of mutual orthogonality of temperature, illumination intensity, TN and TP on MCs is presented in Table 3. The contents of MCs increase continuously during the culture in all treatments. The MCs contents on the 23rd day are highest for all treatments. Treatment group 4 achieves the highest MCs content on the 15th day. The content of MCs increases quickly during the first 15 days and becomes stable subsequently. This might be caused by the short logarithmic phase of *Microcystis* in 4#. The content of MCs on the 23rd day is the highest among all treatments. The MCs content in 6# was the highest, and the reason might be that there are the most algae cells in it. However, although the maximum biomass in 3# is next to that in 6#, the content of MCs is only half of that in 6#. This means that the content of MCs is not only related to biomass, but also other factors.

Synergy Effect of Temperature, Illumination Intensity, Nitrogen and Phosphorus on Biomass of *Microcystis*

To study the effect of degrees of temperature, illumination intensity, nitrogen and phosphorus on *Microcystis* growth, a statistical analysis on the biomass of *Microcystis* was con-

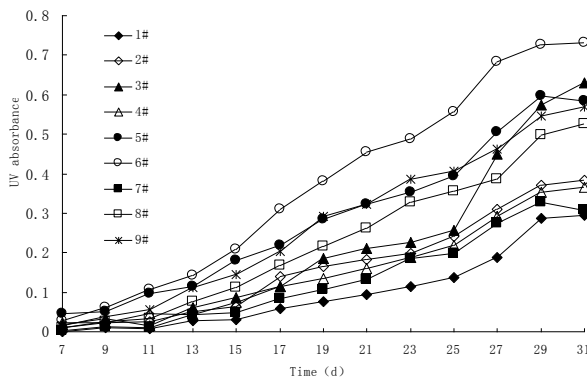
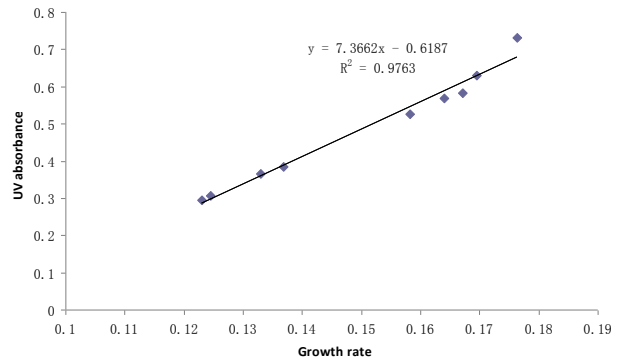
Table 3: Content of MCs ($\mu\text{g}\cdot\text{L}^{-1}$) during 23-day culture.

Number	The 7 th day	The 11 th day	The 15 th day	The 19 th day	The 23 rd day
1#	0.5240	1.7556	4.3031	18.0597	20.8622
2#	0.1937	3.2324	5.8781	12.9239	21.9742
3#	0.1588	2.7712	5.6402	10.0309	15.1562
4#	0.1674	2.3533	27.4981	30.5777	31.5704
5#	0.4844	2.8458	5.8047	10.9054	18.9009
6#	0.2952	3.7680	14.0460	32.1244	46.3429
7#	0.5303	2.0560	6.5060	10.3597	20.8562
8#	0.2407	2.2880	10.8364	17.4097	18.2394
9#	0.5552	2.2081	10.6787	13.5437	15.3474

ducted. The orthogonal test was implemented according to Table 2. The absorbance value of algae solution was tested every other day since 7d, getting variation curves (Fig. 2). All treatment groups enter into the logarithmic phase since 9d and have a long logarithmic phase. The maximum absorbance value of treatment group 6 is significantly higher than those of other treatment groups. The absorbance value of treatment group 1 changes slightly, but absorbance values of treatment group 2, 3, 4 and 7 vary similarly.

The relationship between the final optical density and specific growth rate during the growth of *Microcystis* is presented in Fig. 3. According to regression analysis, the correlation of specific growth rates of different treatment groups and their absorbance values is $y = 7.3662x - 0.6187$ ($R^2 = 0.9763$). Therefore, the growth of *Microcystis* can be represented by the orthogonal analysis based on the final absorbance value.

Statistics were calculated according to the final absorbance values of *Microcystis*. The intuitive analysis results are listed in Table 4. The mean value in Table 4 represents the average absorbance values of each level of different

Fig. 2: Absorbance value of *Microcystis* under different culture conditions.Fig. 3: Correlation between *Microcystis* absorbance and specific growth rate.

factors. For example, T_{11} , T_{21} and T_{31} represent the final absorbance values at 22°C, 25°C and 28°C, respectively. It can be observed that *Microcystis* growth increases with the temperature rise, but the algal density begins to decrease after the temperature increases to a certain level. The content of *Microcystis* increases with the increase of illumination intensity. *Microcystis* growth changes similarly as TN and TP increases, decreasing first and then increasing. The range in Table 4 means the different effect of the factors on algae growth. The higher the range is, the stronger the effect was. As a result, illumination intensity influences the algal growth strongly, followed by temperature, TN and TP.

Illumination provides energies for the cellular metabolism of algae and affects algal growth and reproduction. *Microcystis aeruginosa* is known to respond to light availability with photoacclimation (Wyman et al. 1983). Huisman et al. (1999) reported that algal growth has specific “critical illumination intensity”. For example, in the water column inoculated with *Microcystis* and *Chlorella*, *Microcystis* will be replaced by *Chlorella* gradually below 3,000Lx illumination intensity. However, in natural lakes and reservoirs, *Microcystis* could beat others in the light fight due to its strong buoyancy. When the controlled illumination intensity is lower than the “critical illumination intensity”, algal biomass will decrease significantly. Our experiment also confirmed that very low illumination intensity will inhibit *Microcystis* growth. *Microcystis aeruginosa* is known for its resistance to photoinhibition and high light due to protective pigmentation (Paerl et al. 1983). The temperature has been shown to have a positive effect on the growth of *M. aeruginosa* (Rainer et al. 2016). Temperature affects algal growth directly or indirectly mainly by controlling physicochemical progress, such as the enzymatic reaction of photosynthesis, respiration intensity and decomposition of nutrient substances in water. In this study, it is discovered that algal content increases with the temperature rise, but algal density begins to decrease when

Table 4: Visual analysis table about absorbance values of *Microcystis*.

Number	Environmental factors				Absorbance values
	Temperature (°C)	Light intensity (Lx)	TN (mg.L ⁻¹)	TP (mg.L ⁻¹)	
1	22	200	20	0.4	0.294
2	22	500	60	0.6	0.384
3	22	800	100	1.0	0.631
4	25	200	60	1.0	0.366
5	25	500	100	0.4	0.584
6	25	800	20	0.6	0.7325
7	28	200	100	0.6	0.3075
8	28	500	20	1.0	0.5265
9	28	800	60	0.4	0.568
Mean value T ₁	0.436	0.322	0.518	0.482	
Mean value T ₂	0.561	0.498	0.439	0.475	
Mean value T ₃	0.467	0.644	0.507	0.508	
Range	0.125	0.322	0.079	0.033	

the temperature increased to a certain level. Within the appropriate temperature range, cell activity enhances with the temperature rise. However, after the temperature reaches a certain level, algal metabolism accelerates, which requires more nutrients. Due to the decreasing nutrients in the water, algal cell death rate under high temperature is higher than the reproduction rate and algal growth is inhibited. Zhao et al. (2007) found that water temperature was an important factor in the shift of *Microcystis* and *Oscillatoria* dominance in a simulated, shallow, eutrophic lake. Algal reproduction is closely related to nitrogen and phosphorus concentrations in water (Stockner & Shortreed 1988, Borchardt 1994). Tsukada

et al. (2006) found that P limitation did not occur during the study period. The effect of N was stronger than that of P for blooms of *Microcystis*. The ratio of N/P plays an important role in *Microcystis* growth, and the low N/P ratio could be a consequence of the capacities and rates of uptake of nitrogen and phosphorus by cyanobacteria (Marinho & Sandra 2007).

Synergy Effect of Temperature, Illumination Intensity, Nitrogen and Phosphorus on Toxin Production by *Microcystis*

In the experiment, a statistical analysis of MCs per unit cell was conducted on the 23rd day. Intuitive analysis results are

Table 5: Visual analysis table about single-cell toxin production by *Microcystis*.

Number	Environmental factors				Toxin production of single cell (fg.a ⁻¹)
	Temperature (°C)	Light intensity (Lx)	TN (mg.L ⁻¹)	TP (mg.L ⁻¹)	
1	22	200	20	0.4	2.5996
2	22	500	60	0.6	1.6429
3	22	800	100	1.0	0.8009
4	25	200	60	1.0	2.3560
5	25	500	100	0.4	0.8290
6	25	800	20	0.6	1.8390
7	28	200	100	0.6	2.5280
8	28	500	20	1.0	0.9426
9	28	800	60	0.4	0.7326
Mean value T ₁	1.681	2.495	1.794	1.387	
Mean value T ₂	1.675	1.138	1.577	2.003	
Mean value T ₃	1.401	1.124	1.386	1.367	
Range	0.280	1.371	0.408	0.636	

listed in Table 5. The high MCs content per unit cell is in 1, 4 and 7 #, which indicated that low illumination intensity is beneficial for toxin production of *Microcystis*. Toxin production per unit cell of treatment group 9 is only next to that of treatment group 3, indicating that other factors could compensate for inadequate phosphorus to a certain extent. This reflects that there's mutual compensation among factors. Furthermore, illumination intensity affects the toxin production of *Microcystis* mostly, followed by TP, TN and water temperature.

In the experiment, levels of four factors are shown in the horizontal coordinate and their meaning is presented by the toxin production per unit cell at the vertical coordinate (Fig. 4). Four curves in Fig. 4 show the effect of temperature, illumination intensity, TN and TP on MCs content per unit cell of *Microcystis*. Effect of TP on toxin production per unit cell of *Microcystis* increases firstly and then decreases, indicating that there's an optimum P concentration within the selected range for *Microcystis* to produce MCs. In this experiment, it was concluded that 0.6 mg.L^{-1} is the optimum P concentration for the production of MCs. MCs content per unit cell of *Microcystis* increases with the decrease of illumination intensity. The effect of temperature and TN is similar to that of illumination intensity. In this study, the optimum illumination intensity, temperature and TN for *Microcystis* to produce toxin are 200 Lx , 22°C and 20 mg.L^{-1} , respectively.

According to the orthogonal test results, low illumination intensity could facilitate toxin production of *Microcystis*. This agrees with the research results of Rapala et al. (1997). Meanwhile, the effect of illumination intensity on toxin production is affected by temperature. With the increase of TP,

MCs show an inverse "V-shaped" variation. Low TP could accelerate MCs production. This echoes with many results (Vonshak et al. 1994, Christina & Pedro 1994, Kameyama et al. 2002). The results of this study showed that there was a slight increase in MCs production at low temperature and a slight decrease in toxicity at high temperature, similar to the results from the studies of Watanabe & Oishi (1985), Sivonen (1990) and Gilbert (1996). With limited phosphorus, both isomers of MCs and MCs increase. Nitrogen is an essential element for MCs synthesis. The proportion of nitrogen in MCs molecular structure was up to 14% (Botes et al. 1985). However, based on our orthogonal test results, N influences MCs slightly. Orr & Jones (1998) found a linear relationship between cell division rate and total MCs production rate in *Microcystis* solution. MCs content per unit cell keeps constant during the cell cycle even though both total MCs content in algal solution and cell concentration increase with the increase of TN in culture medium. Therefore, it is speculated that the effect of TN on total MC may be insignificant because nitrogen changes total MCs content in *Microcystis* solution by influencing the algae cell growth, but there is no direct impact on MCs content per unit cell. Meanwhile, the orthogonal test results reveal that the optimum growth condition and the optimum toxin production condition of *Microcystis* are different from each other. Abundances of toxic strains of *Microcystis* have been specifically enhanced by additions of inorganic N (Davis et al. 2010).

CONCLUSIONS

Variation trends of *Microcystis* biomass and extracellular MCs are analyzed through a 29-day orthogonal test of tem-

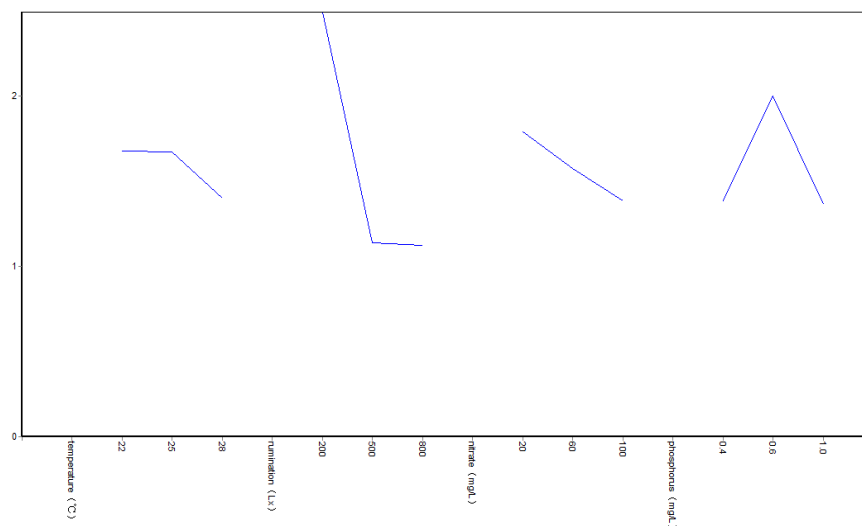


Fig. 4: Orthogonal test of temperature, illumination intensity, nitrogen and phosphorus.

perature, illumination intensity, nitrogen and phosphorus. The conclusions are as following:

1. According to the intuitive analysis on optical density of *Microcystis* solution, illumination intensity influences *Microcystis* growth mostly, followed by temperature, TN and TP.
2. The MCs content in 6# was the highest, and the reason might be that there are the most algal cells in it. However, although the maximum biomass in 3# is next to that in 6#, the content of MCs is only half of that in 6#. This means that the content of MCs is not only related to biomass, but also other factors
3. Illumination intensity influences toxin production of *Microcystis* strongly, followed by TP, TN and water temperature successively. With the increase of TP, MCs content per unit cell increases firstly and then decreases. In this study, the optimum TP for toxin production of *Microcystis* is 0.6 mg.L⁻¹. Toxin production per unit cell is inversely proportional to illumination intensity, temperature and TN. The optimum illumination intensity, temperature and TN for toxin production of *Microcystis* are 200Lx, 22°C and 20 mg.L⁻¹, respectively.

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