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Nitrogen limitation significantly reduces the competitive advantage of toxic *Microcystis* at high light conditions

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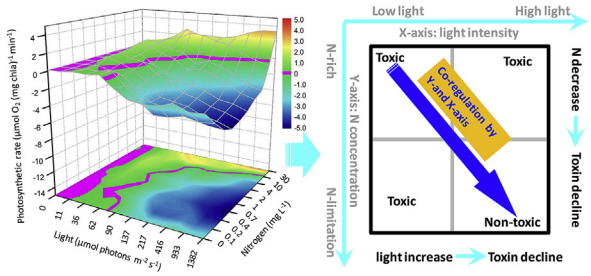
HIGHLIGHTS

- N-rich or low light favors the competitive advantage of toxic *Microcystis*.
- N-limitation combined with high light promotes the dominance of non-toxic *Microcystis*.
- The interactive effect of N-limitation and high light reduces the MCYST cellular quota.
- The competition processes above mentioned were promoted by rising temperature.

GRAPHICAL ABSTRACT



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ABSTRACT

Microcystis is a notorious cyanobacterial genus due to its rapid growth rate, huge biomass, and producing toxins in some eutrophic freshwater environments. To reveal the regulatory factors of interspecific competition between toxic and non-toxic *Microcystis*, three dominant *Microcystis* strains were selected, and their photosynthesis, population dynamics and microcystins (MCYST) production were measured. The results suggested that nitrogen-limitation (N-limitation) had a greater restriction for the growth of toxic *Microcystis* than that of non-toxic *Microcystis*, especially when cultured at high light or high temperature based on the weight analysis of key factors. Comparison of photosynthesis showed that low light or N-rich would favor the competitive advantage of toxic *Microcystis* while high light combined with N-limitation would promote the competitive advantage of non-toxic *Microcystis*, and these two competitive advantages could be further amplified by temperature increase. Mixed competitive experiments of toxic and non-toxic *Microcystis* were conducted, and the results of absorption spectrum (A_{485}/A_{665}) and *q*PCR (real-time quantitative PCR) suggested that the proportion of toxic *Microcystis* and the half-time of succession process were significantly reduced by 69.4% and 28.4% ($p < 0.01$) respectively by combining N-limitation with high light intensity than that measured under N-limitation condition. N-limitation led to a significant decrease of MCYST cellular quota in *Microcystis* biomass, which would be further decreased to a lower level by the high light. Based on above mentioned analysis, to decrease the MCYST production of *Microcystis* blooms, we should control nutrient, especial nitrogen through pollutant intercepting and increase the light intensity through improving water transparency.

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1. Introduction

Microcystis is a notorious cyanobacterial genus due to its rapid growth, huge biomass, and ability of produce toxins of certain species, and it is the dominant genus in summer and can form serious blooms in some large shallow eutrophic lakes of China such as Lake Taihu (Zhu et al., 2016; Harke et al., 2016). The growth of *Microcystis* is affected by many environmental favors such as N/P (Zhu et al., 2015), nutrients level, water temperature, light intensity, turbulence, and so on (Paerl and Otten, 2013), and its blooms are harmful to aquatic animals and human beings due to the production of cyanotoxins including MCYST (Carmichael, 1992; Paerl and Otten, 2013). Some study reported that low N/P ratio or relative N-limitation could increase the MCYST concentration of water column (Orihel et al., 2012), but others showed that MCYST concentrations in lakes were positively correlated with N/P ratios (Beversdorf et al., 2015). These previous contradictory conclusions indicated that (1) the effects of nutrients on MCYST concentrations were uncertain and (2) MCYST concentrations might be regulated by the interactive effects of various environmental factors rather than a single factor as suggested by Tonk et al. (2009).

MCYST concentration in water column is not only determined by the biomass of total *Microcystis* but also by the ratio of toxic species to the non-toxic ones. Therefore, to precisely evaluate the water quality safety, it is necessary to focus on the population dynamics and dominance of toxic *Microcystis* in community structure. The biomass and proportion of MCYST-producing *Microcystis* varied with seasons, and are closely related to the blooming stage. Li et al. (2014a) reported that *Microcystis* blooms were composed of mixed species, and the percentage of toxic genotypes was between 4.4% and 64.3% in Lake Taihu, which gradually increased with the formation of bloom and suddenly declined in the late period of bloom. Generally, toxic *M. aeruginosa* was dominant at the early stage of bloom formation, and this dominance was gradually replaced by nontoxic *M. wesenbergii* at the later period of blooms (Yamamoto and Nakahara, 2009; Li et al., 2013).

Many researchers attempted to reveal the key factors driving the population dynamics of toxic cyanobacteria in eutrophic lakes, and they mainly focused on seasonal variations in temperature and concentration of various nutrients (Briand et al., 2008; Davis et al., 2009). Some studies have shown that nutrient concentration and temperature were the main factors controlling the MCYST dynamics (Harke et al., 2016; Maliaka et al., 2018), and high ratios of N/P and C/N were closely related to the toxic phase of *Microcystis* bloom (Beversdorf et al., 2015), which indicated that N-rich or C-limitation would result in the dominance of toxic *Microcystis*. And this competitive advantage of toxic *Microcystis* may be further enhanced with increasing temperature (Conradie and Barnard, 2012). It has been reported that both of the biomass of toxic *Microcystis* and its ratio to non-toxic *Microcystis* were higher in tropical reservoirs under suitable conditions of sunlight, nutrients and temperature than that in temperate water bodies (Te and Gin, 2011).

Except for nutrients, light also has a significant impact on the production of MCYST in toxic *Microcystis*, and relatively high light intensity below saturated light intensity generally leads to higher MCYST production (Kaebernick et al., 2000; Wiedner et al., 2003). MCYST are located on the thylakoid membrane of *Microcystis* cells (Young et al., 2005), indicating that MCYST may play a major role in the regulation of photosynthesis. There are some significant differences in photosynthesis and growth characteristics between MCYST-producing *Microcystis* and non-toxic strains, and UVB radiation may be in favor of the former (Ding et al., 2013; Wang et al., 2015). Phelan and Downing (2011) reported that toxic *Microcystis* could survive and maintain normal growth while the non-toxic *Microcystis* including wild strains and mutant strains died immediately due to photo-inhibition when exposed to high light, and they also suggested that the growth rate of toxic *Microcystis* had a positive correlation with the intracellular MCYST cellular quota.

All of these previous researches seem to show that the population dynamics of toxic *Microcystis* are regulated by a variety of environmental factors rather than by a single environmental factor. In this case, our question arises: when one environmental factor, such as high light, is beneficial to the dominance of toxic *Microcystis* while another environmental factor, such as low nitrogen, is beneficial to the dominance of non-toxic *Microcystis*, which kind of *Microcystis* is the ultimate winner? To answer this question, we need to understand the co-regulation of environmental factors. However, as so far, few studies have focused on the interactive effects of light, nutrients and temperature on the competition between toxic and non-toxic *Microcystis*.

In this study, the dominant toxic and non-toxic *Microcystis* strains isolated from Lake Taihu were used as experimental objects. We will study the effects of temperature, nutrients and light on the competition between toxic and non-toxic *Microcystis*, and try to determine which the key driver is and its interactive effect with other environmental factors, also quantify the effective weight of each factor in the competition. According to the above research contents, we can reveal the population regulation mechanism of toxic *Microcystis* and predict the concentration dynamics of MCYST in water column. This study will provide a scientific basis for the development of lake management strategies and effective control of toxic *Microcystis*.

2. Materials and methods

2.1. Strains and culture conditions

Toxic *Microcystis aeruginosa* (*M. aer*-T) and non-toxic *Microcystis wesenbergii* (*M. wes*-N) are two dominant *Microcystis* species during the bloom period in summer, also there are some natural mutant strains of toxic *Microcystis* (e.g. non-toxic *Microcystis aeruginosa*, abbreviated as *M. aer*-N), which lost the ability of MCYST-production. The dynamics of MCYST concentrations in lakes are mainly caused by interspecific succession between these different toxic *Microcystis*. Therefore, above three kinds of *Microcystis* including *M. aer*-T, *M. wes*-N and the non-toxic natural mutant of *M. aer*-N were isolated from Lake Taihu and selected as experimental strains. These three strains were kindly provided by the Freshwater Algae Culture Collection at the Institute of Hydrobiology, Chinese Academy of Sciences (FACHB Collection) and their registration numbers were FACHB1322 (*M. aer*-T), FACHB1334 (*M. wes*-N) and FACHB1343 (*M. aer*-N), respectively. They were cultured with BG-11 medium (Rippka et al., 1979) at a 12:12 light: dark cycle with 30 μmol photons m^{-2} s^{-1} light provided by cool white fluorescent tubes at 25 ± 1 °C. Then, they were collected in the period of exponential phase at 9600×*g* for 8 min with Hitachihigh-speed refrigerated centrifuge (CR21GII, Hitachi Koki Co., Ltd., Tokyo, Japan), and pelleted cells were washed three times with nitrogen-free and phosphate-free BG-11 medium. Subsequently, cultures were phosphate starved in the abovementioned phosphate-free BG-11 for 72 h at a 12:12 light: dark cycle with an irradiance of 30 μmol photons m^{-2} s^{-1} at 25 ± 1 °C. These starved *Microcystis* were re-inoculated to fresh mediums with various treatments, which then were used for the following experiments of photosynthesis analysis and mixed population competition.

2.2. Analysis of mixed population competition

The starved cells were re-inoculated into 150 ml glass flasks

filled with 100 ml of modified BG-11 medium with various nutrient concentrations and cultured at different temperatures (shown as in Table 1).

Two *Microcystis* species, *M. aer*-T and *M. wes*-N, were used to investigate the competition between the toxic and non-toxic *Microcystis*. According to previous research, some strains may have different light absorption due to the pigment contents (Kardinaal et al., 2007), we measured the absorption spectrum between 350 nm and 750 nm (Kardinaal et al., 2007), and found that there was a significant difference in the A_{485}/A_{665} value between *M. aer*-T and *M. wes*-N, and the A_{485}/A_{665} value of each strain did not seem to vary with the culture conditions. Therefore, in this study, A_{485}/A_{665} was chosen as the indictor of the ratio of *M. aer*-T and *M. wes*-N. The two starved species were mix-inoculated into above mentioned medium with five different treatments shown in Table 1, and the algal cells were collected every two days to measure the A_{485}/A_{665} until the end of experiment on the 24th day, which shown in Fig. 7. The non-linear regressions of A_{485}/A_{665} to days were fitted by Boltzmann formula, and the half-time of succession was calculated as shown in Fig. 7C. Then, the toxic *Microcystis* was calculated with formula as following:

$$R_{Toxic} = (R_{F1322} - R_{F1334}) / R_{F1322} * 100\% \quad (1)$$

In this formula, R_{Toxic} is the ratio of toxic *Microcystis*, R_{F1322} and R_{F1334} are the A_{485}/A_{665} of *M. aer*-T and *M. wes*-N, respectively.

Three strains of *Microcystis* (*M. aer*-T, *M. wes*-N and *M. aer*-N) were also used to investigate the competition between the toxic and non-toxic *Microcystis*. The starved *Microcystis* were mix-inoculated into above mentioned medium with five different treatments shown in Table 1, and the initial inoculated cell density of the three strains were respectively 5.0×10^4, 2.5×10^4 and 2.5×10^4 cell mL^{-1} so as to ensure the ratio of toxic and non-toxic *Microcystis* was 1: 1. Then, the cells were collected on the 14th day of culture and the population competition between toxic and non-toxic *Microcystis* were quantified and analyzed using the method of real-time quantitative PCR (qPCR) shown as following.

2.3. Photosynthetic oxygen evolution

The starved cells were then re-inoculated into 150 ml glass flasks filled with 100 ml of modified BG-11 medium containing various nutrient concentrations and cultured at different temperatures (T) and light intensity (L) as shown in Table 2. The concentrations of N and P shown in Table 2 were adjusted by $NaNO_3$ and KH_2PO_4 respectively. Potassium ions and pH in all modified culture medium were adjusted to be consistent with basic BG-11 medium (BG-11 with $N = 30\ mg\ L^{-1}$ and $P = 1.0\ mg\ L^{-1}$) by adding 0.1 mol L^{-1} KCl solution and 0.1 mol L^{-1} HCl. The initial inoculated densities of the three *Microcystis* were 5.0×10^4 cell mL^{-1}.

Microcystis cells cultured under above-mentioned various conditions were collected by centrifugation at exponential growth phase (on the 8th day in this study), and then used for the measurement of photosynthesis. Photosynthetic oxygen evolution rate of cultures was measured with a Clark-type oxygen electrode (Chlorolab-2, Hansatech, UK) according to previous research (Wang

Table 1
The culture conditions of mixed population competition experiments.

Experiment groups	N concentration (mg/L)	P concentration (mg/L)	Light intensity (μmol·photons·m^{-2}·s^{-1})	Temperature (°C)
control	30.0	1.0	60	25
HL	30.0	1.0	100	25
LN	1.0	1.0	60	25
LN & HL	1.0	1.0	100	25
LN & HL & HT	1.0	1.0	100	33

Notes: HL, high light; LN, low nitrogen; LN & HL, low nitrogen combined with high light; LN & HL & HT, low nitrogen combined with high light and high temperature.

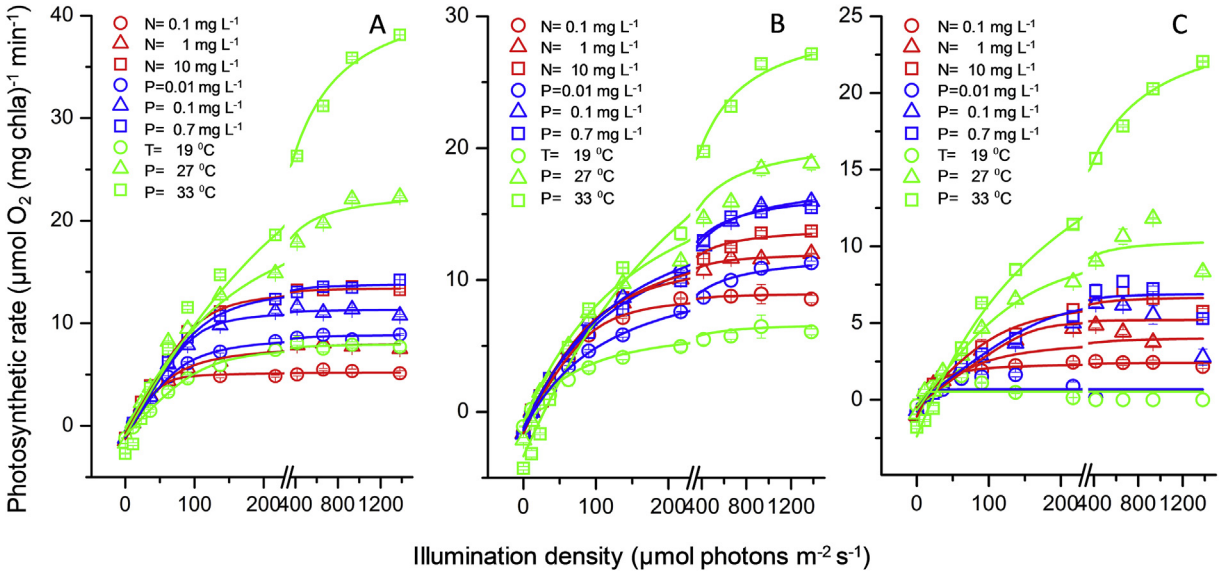

Fig. 1. Photosynthetic light-response curves (P-I curves) of (A) *M. aer*-T, (B) *M. wes*-N and (C) *M. aer*-N which cultured in different Nitrogen concentrations (0.1 mg L^{-1}, 1 mg L^{-1} and 10 mg L^{-1}), phosphorus concentrations (0.01 mg L^{-1}, 0.1 mg L^{-1} and 0.7 mg L^{-1}) and temperature (19 °C, 27 °C and 33 °C). Various steps of light intensity were set as following: 0, 11, 36, 62, 90, 137, 217, 416, 933, 1382 μmol photons m^{-2} s^{-1}.

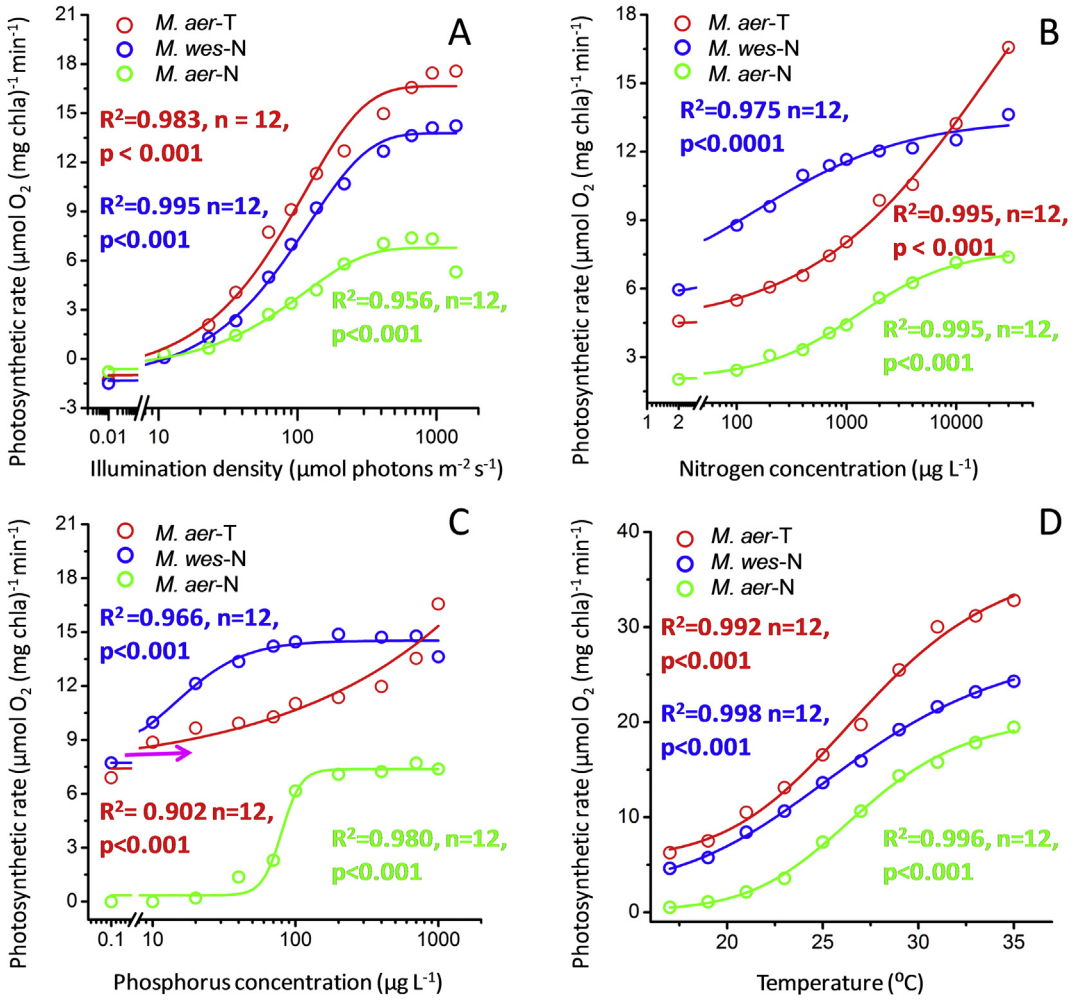

Fig. 2. Rate of photosynthesis of *M. aer*-T, *M. wes*-N and *M. aer*-N under conditions of (A) light-intensity steps including 0, 11, 36, 62, 90, 137, 217, 416, 933 and 1382 μmol photons m^{-2} s^{-1}; (B) Nitrogen concentration gradients including 0, 0.1, 0.2, 0.4, 0.7, 1, 2, 4, 10 and 30 mg L^{-1}; (C) Phosphorus concentration gradients including 0.01, 0.02, 0.04, 0.07, 0.1, 0.2, 0.4, 0.7 and 1 mg L^{-1}; (D) Temperature steps including 17 °C, 19 °C, 21 °C, 23 °C, 25 °C, 27 °C, 29 °C, 31 °C, 33 °C and 35 °C.

et al., 2015), and the conditions set for the measurement were consistent with those for their cultures. The photosynthetic light-response curves (P-I curves) of *Microcystis* cultured under conditions of T = 25 °C, L = 60 μmol·photons m^{-2} s^{-1}, N = 30 mg L^{-1} and P = 1.0 mg L^{-1} were recorded with increasing light intensities (dark, 11, 23, 36, 62, 90, 137, 217, 416, 662, 933 and 1382 μmol·photons·m^{-2}·s^{-1}). All data of photosynthesis vs. irradiance were fitted using nonlinear regression in SPSS statistical software (version 11.5, USA), using the following equation (1):

$$A = \frac{\varphi.Q + A_{max} - \sqrt{\left\{(\varphi.Q + A_{max})^2 - 4\theta.\varphi Q.A_{max}\right\}}}{2\theta} - R_{day} \quad (2)$$

where A is the net photosynthetic rate (μmol O_2 mg^{-1}·chl-*a* min^{-1}), φ is the apparent quantum efficiency, A_{max} is the maximum photosynthetic efficiency, θ is the angle value of non-linear fitting equation, R_{day} is the respiration rate under light condition, and Q is light intensity of various illumination steps. All the parameters, including P_{max}, can be read from this fitting equation.

The inhibition ratio of photosynthetic quantum efficiency (IRPQ) was calculated with the following formula (2):

$$IRPQ = (P_{suitale}\text{-}P_{stress}) / P_{suitable} * 100\% \quad (3)$$

Here, P_{stress} represents the photosynthetic rate at N stress (or P stress or low temperature stress), and $P_{suitale}$ represents the photosynthesis at N rich (or P rich or suitable temperature), the calculated results are shown in Fig. 3.

2.4. Measurement of chlorophyll-a concentration

Chlorophylla (chla) was used to calculate the photosynthetic oxygen evolution rates (μmol O_2 (mg chla)$^{-1}$ min^{-1}, i.e., photosynthetic rate) of *Microcystis* cultures. After the measurement of photosynthetic rates, the cells of *Microcystis* samples were collected by centrifuging at 3000×*g* at 4 °C for 15 min in a Hitachi high-speed refrigerated centrifuge. The supernatant was discarded and the pellets were extracted with 90% acetone for 24 h at 4 °C in dark. Chla concentration was measured with a spectrophotometer and calculated using the formula m_{Chla} (mg L^{-1}) = 11.85*A_{664}–1.54*A_{647} − 0.08*A_{630} (Jeffrey and Humphrey, 1975).

2.5. Weight comparison of environmental factors

To clarify what is the key factor in determining photosynthesis under different culture conditions, we compared actual values with

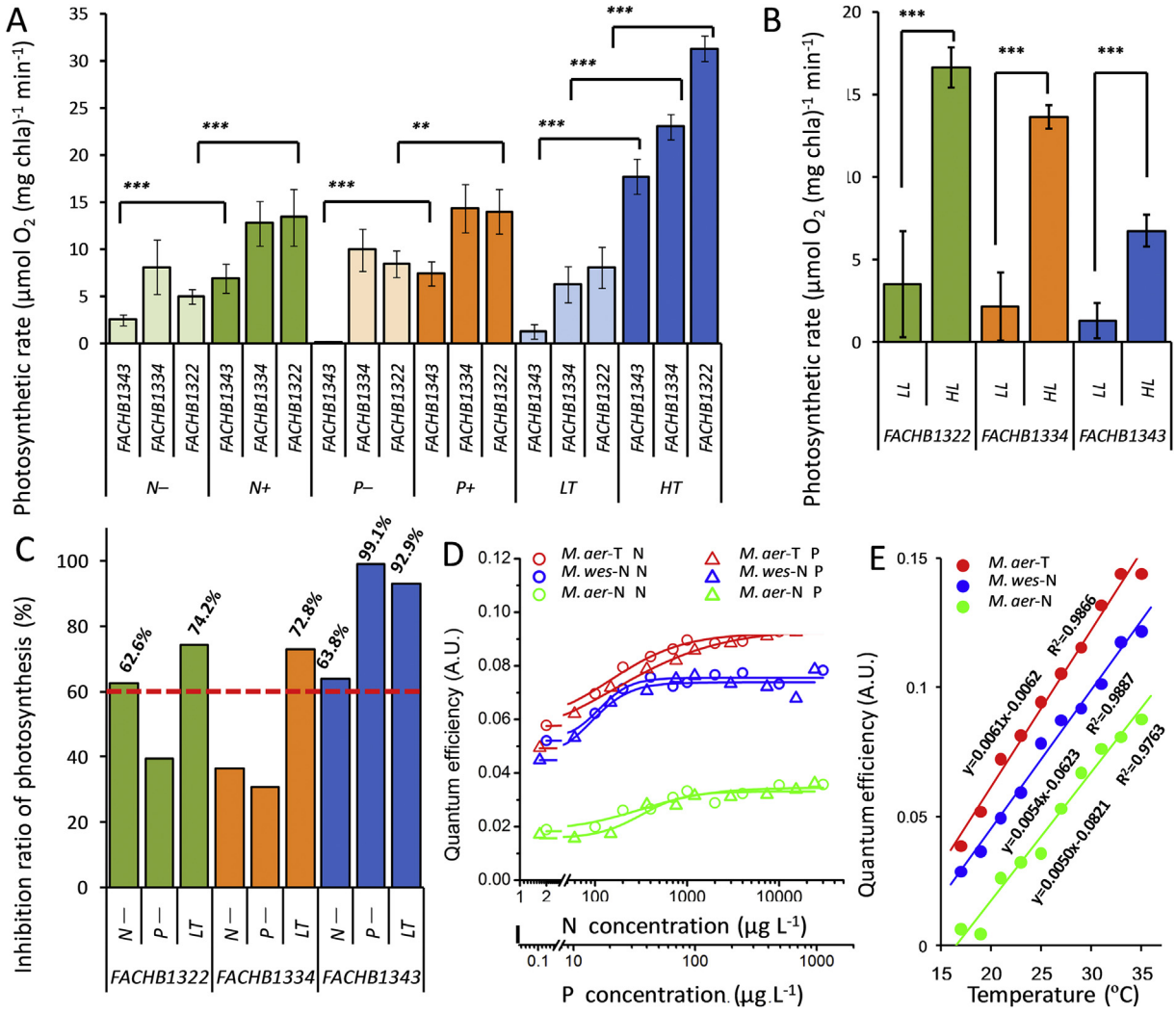

Fig. 3. The comparison of quantum efficiency and photosynthetic rate of *M. aer*-T, *M. wes*-N and *M. aer*-N under various treatment conditions. Specifically, these comparison include (A) Rate of photosynthesis under conditions of low N concentrations of 0–0.4 mg L^{-1} (N–), low P concentrations of 0–0.04 mg L^{-1} (P–), low temperature stress of 17–21 °C (LT), N-rich concentrations of 4–30 mg L^{-1} (N+), P-rich concentrations of 0.4–1 mg L^{-1} (P+) and high temperature of 31–35 °C (HT); (B) Rate of photosynthesis when cultured under conditions of low-light of 11–62 μmol photons m^{-2} s^{-1} (LL) and high-light 416–1382 μmol photons m^{-2} s^{-1} (HL); (C) calculation of photosynthetic inhibition which caused by above mentioned N–, P– and LT treatments; (D) Nonlinear fitting curves of quantum efficiencies with gradient increase of nitrogen and phosphorus concentrations; (E) linear regression of quantum efficiencies with gradient increase of temperatures. The level of significance of differences between these various treatments are indicated by *, **, *** for $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

the maximal values in specific columns and rows, and then calculated the limiting effects of temperature, light and nutrients. The weight analysis method in this study can intuitively show which the main limitation factor in the co-regulation of two environmental factors is. The weight is equal to the difference value of above calculated limitation effects. Firstly, the measured values of photosynthesis are arranged according to rows (X-axis) and columns (Y-axis), and then the weight is calculated with these values in different rows and columns. Weight is a relative parameter that used to quantify the specific effect of one factor on the algal photosynthesis when compared with that of another factor. The weight of various gradients of factors on the photosynthesis is calculated with the following formulas (4)–(7),

$$Lim(\mathrm{Y})_{i\cdot j} = 1 - P_{i\cdot j} / P_{\max\cdot j} \quad (4)$$

$$Lim(\mathrm{X})_{i\cdot j} = 1 - P_{i\cdot j} / \mathrm{P}_{i\cdot max} \quad (5)$$

$$W(\mathrm{Y})_{i\cdot j} = (Lim(\mathrm{Y})_{i\cdot j} - Lim(\mathrm{Y})_{i\cdot j}) / [(1 - Lim(\mathrm{Y})_{i\cdot j}) + (1 - Lim(\mathrm{Y})_{i\cdot j})] \quad (6)$$

$$W(\mathrm{X})_{i\cdot j} = 1 - W(\mathrm{Y})_{i\cdot j} \quad (7)$$

In above formulas, $Lim(\mathrm{Y})_{i\cdot j}$ and $Lim(\mathrm{X})_{i\cdot j}$, are the limitation effects of Y-axis variables (i.e., temperatures, in columns, in Supplement 2) and X-axis variable (i.e., light intensities, in rows, in Supplement 2) on the photosynthetic rate ($P_{i\cdot j}$) of *Microcystis*, respectively; $P_{i\cdot j}$ is the crossing point of photosynthetic rate in column i on X-axis and row j on Y-axis; $W(\mathrm{Y})_{i\cdot j}$, and $W(\mathrm{X})_{i\cdot j}$ are respectively the relative weight of Y-axis variable and X-axis variable on the photosynthetic rate of *Microcystis*; $P_{\max\cdot j}$ and $\mathrm{P}_{i\cdot max}$ are the maximal value of the photosynthetic rate in column j and row i, respectively. Here, Y-axis indicates vertical column, namely the changes of photosynthesis along with vertical-layout increasing nutrient concentrations which as shown in Fig. 6 (or along with vertical-layout increasing temperature in Supplement 2); and X-

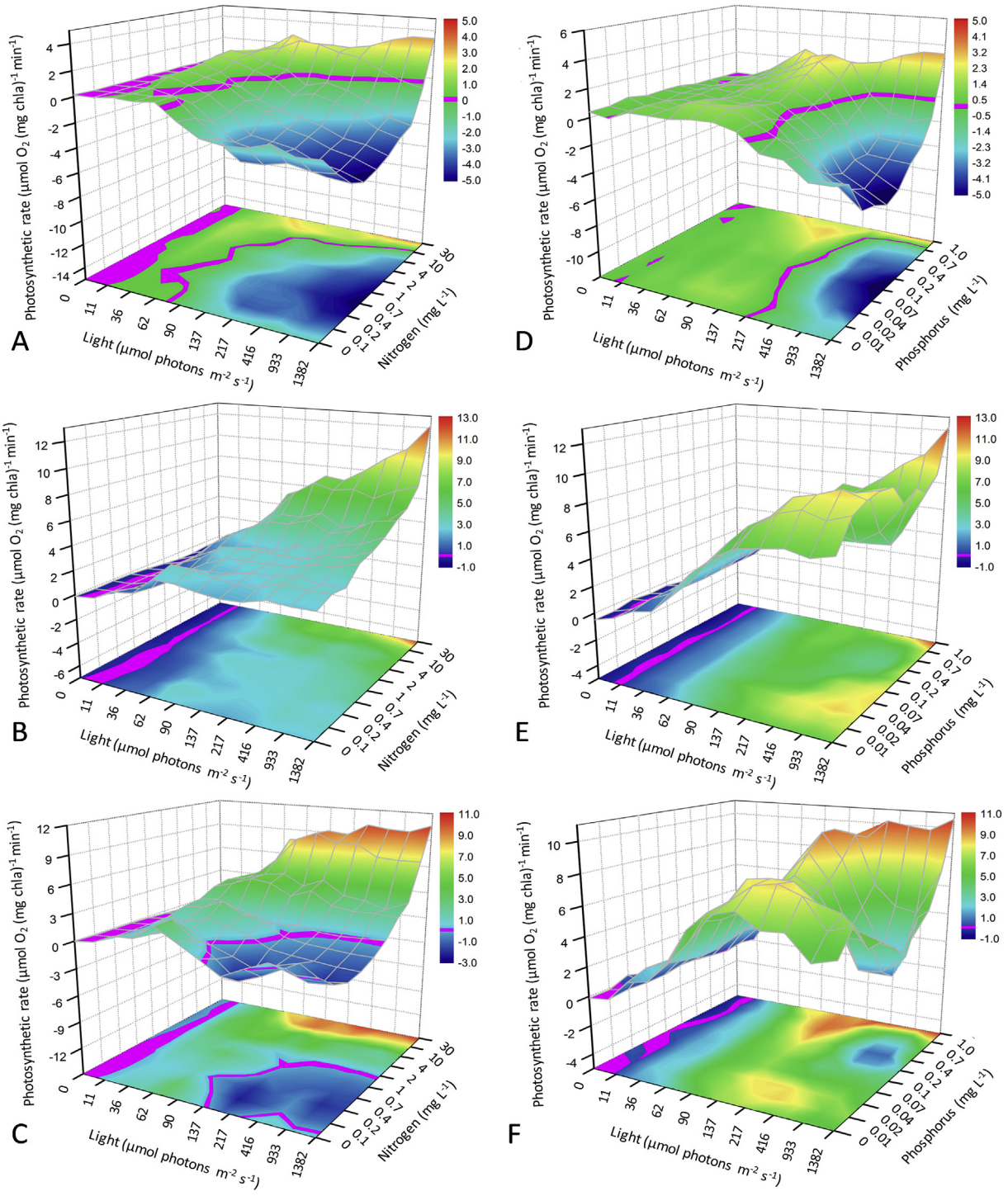

Fig. 4. The photosynthetic difference value (DP-value) between toxic (*M. aer*-T) and non-toxic (*M. wes*-N and *M. aer*-N) *Microcystis* under various conditions of different light intensities and nutrient (nitrogen and phosphorus) concentrations. Specifically, Figure A–C are combination culture conditions of various light intensities and various nitrogen concentrations, and Figure D–F are combination culture conditions of various light intensities and various phosphorus concentrations. There are 100 data points (10 × 10) were shown in every figure of Figure A–F, and each data point represents the DP-value at a specific combination of light intensity (× 10) and nutrient concentration (× 10). Figure A and D are the calculated results of the photosynthetic competitions suggested by DP-value between *M. aer*-T and *M. wes*-N; Figure B and E showed the competitions of photosynthesis suggested by DP-value between *M. aer*-T and *M. aer*-N; Figure C and F are the competitions of the photosynthesis among *M. aer*-T, *M. wes*-N and *M. aer*-N.

axis indicates horizontal row, namely the changes of photosynthesis along with horizontal-layout increasing light intensities or temperature which as shown in Fig. 6.

2.6. DNA extraction and real-time quantitative PCR

Since it is difficult to distinguish toxic *Microcystis* from non-toxic *Microcystis* by morphology, the molecular biological method was

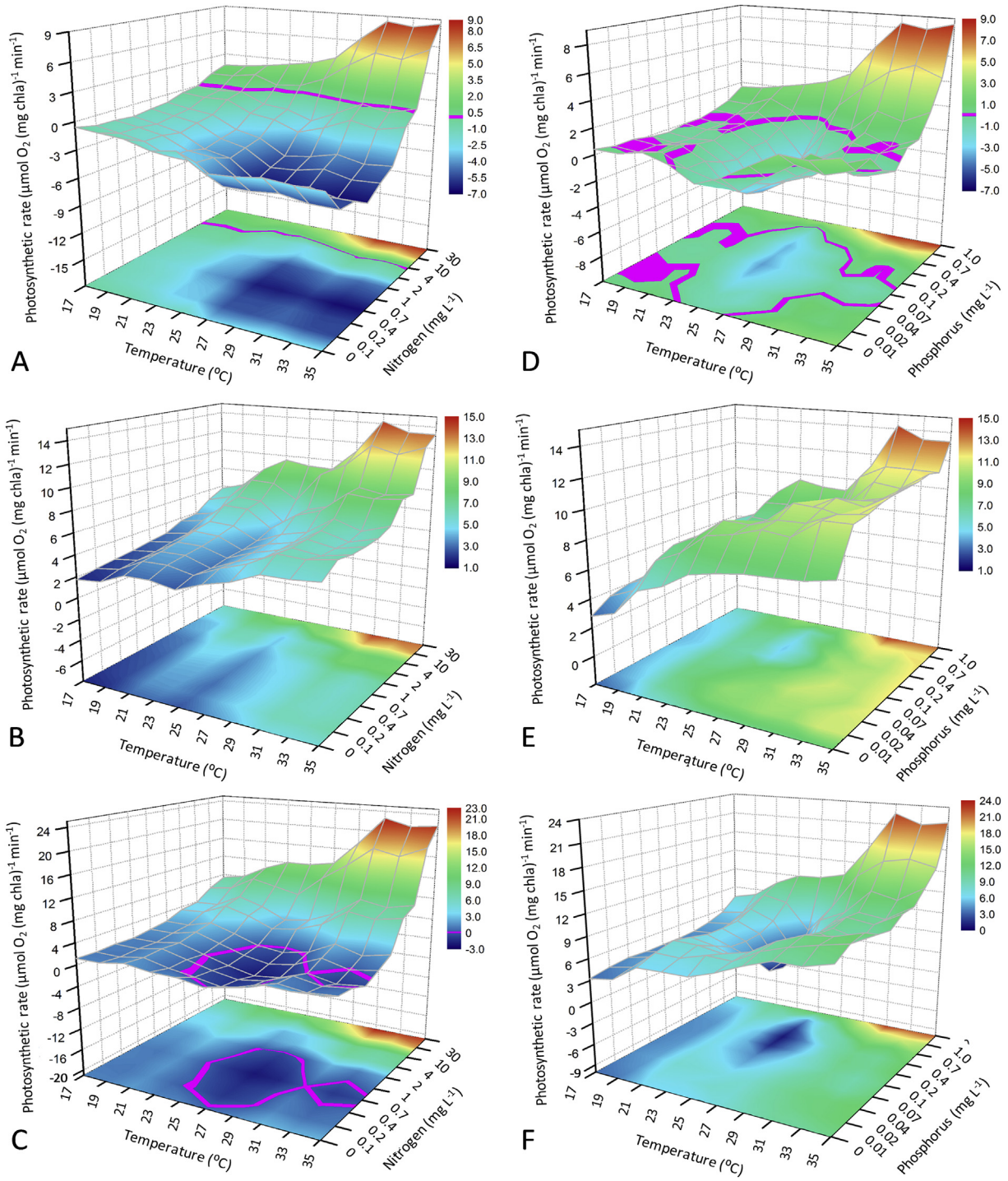

Fig. 5. The photosynthetic difference value (DP-value) between toxic (*M. aer*-T) and non-toxic (*M. wes*-N and *M. aer*-N) *Microcystis* under various conditions of different temperatures and nutrient (nitrogen and phosphorus) concentrations. Specifically, Figure A–C are combination culture conditions of various temperature and various nitrogen concentrations, and Figure D–F are combination culture conditions of various temperature and various phosphorus concentrations. There are 100 data points (10 × 10) were shown in every figure of Figure A–F, and each data point represents the DP-value at a specific combination of temperature (× 10) and nutrient concentration (× 10). Figure A and D are the calculated results of the photosynthetic competitions suggested by DP-value between *M. aer*-T and *M. wes*-N; Figure B and E show the competitions of photosynthesis suggested by DP-value between *M. aer*-T and *M. aer*-N; Figure C and F are the competitions of photosynthesis among *M. aer*-T, *M. wes*-N and *M. aer*-N.

applied to identify toxic algal cells and quantify their proportion in total cells due to DNA of toxic *Microcystis* instead of non-toxic ones contains MCYST synthase genes (*mcyA-F*). *Microcystis* cells were collected aseptically using filtration method and then processed for genomic DNA extraction using a standard phenol chloroform procedure after thawing the frozen filters at room temperature (Sambrook and Russell, 2001) combined with a phenol extraction method used in qPCR analysis of plankton (Rinta-Kanto et al., 2005). Briefly, *Microcystis* cells were suspended in 100 ml lysis buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.2% sodium dodecyl sulfate), and added proteinase K (Qiagen Inc., Valencia, CA) to a final concentration of 50 mg ml^{-1}.

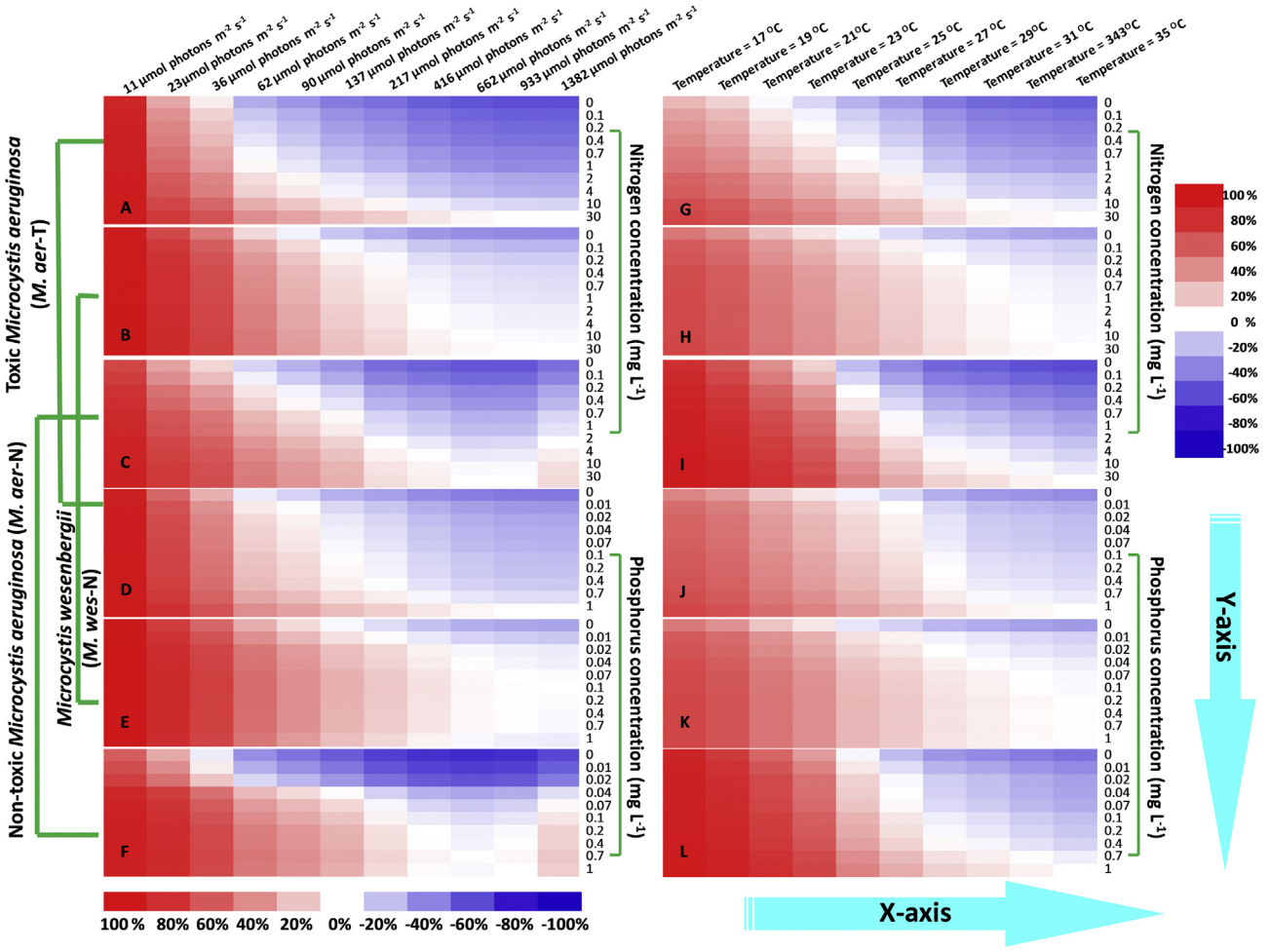

Fig. 6. The weight analysis of three factors (light intensity, nutrient concentration and temperature) in the regulation process of photosynthesis. The color of each check laying the crossing of one factor step along X-axis and another factor step along Y-axis suggests the main contributed factor, and the red checks suggest it has a larger contribution for the factors along X-axis while the blue checks indict the factors along Y-axis have a bigger weight. Figure A–C show the weight percentage of specific light intensity (X-axis) or specific nitrogen concentration (Y-axis); Figure D–F show the weight percentage of specific light intensity (X-axis) or specific phosphorus concentration (Y-axis); Figure G–I show the weight percentage of specific temperature (X-axis) or specific nitrogen concentration (Y-axis); Figure J–L show the weight percentage of specific temperature (X-axis) or specific phosphorus concentration (Y-axis). The test object in Figure A, D, G, J is *Microcystis M. aer*-T, while in Figure B, H, E, K and Figure C, I, F, L, the detected algae is *M. wes*-N and *M. aer*-N, respectively.

Real-time PCR assays were used to quantify three genetic elements, the MICR16S and *mcyD* regions according to Neilan et al. (1997), Rinta-Kanto et al. (2005), Baxa et al. (2010), and Li et al. (2014a) and Li et al. (2017) with some modifications. Genomic DNA of *M. aeruginosa* PCC7806 (toxic) was used as external standard to determine copy numbers of MICR16S and *mcyD*. Firstly, *M. aeruginosa* 7806 with known quantity of cells was filtered through 0.22-μm-pore-size filters, and then, the DNA was extracted according to the method described above. The DNA concentration (ABS_{260} nm) and purity (ABS_{260} nm/ABS_{280} nm) were determined by spectrophotometer. A series with six data points of 10-fold dilutions of genomic DNA of the standard strain *M. aeruginosa* PCC 7806 were prepared and these dilutions were amplified with MICR16S and *mcyD* primers shown in Table 3. Copy numbers of three genes on the basis of DNA were evaluated according to Vaitomaa et al. (2003). Cell abundance was inferred from the standard curve (cell abundance vs C_t) determined in each assay. Amplifications and quantifications were performed using Bio-Rad real-time PCR (CFX Connect, California, USA). The qPCR reactions on a 96-well plate (ABI, CA, USA) were performed in 50 μL total volume (Rinta-Kanto et al., 2005).

2.7. MCYST cellular quota analysis of Microcystis

MCYST of algal sample were extracted and measured according to methods reported by Wang et al. (2012a) with some modifications. Twenty ml cultures were sampled and *Microcystis* cells were collected by centrifugation on the 14th day during the growth period, then the concentration of MCYST was extracted with C18 extraction cartridge (Sep-Pak®, Waters) and analyzed by high-performance liquid chromatography (HPLC, LC-20A system, Shimadzu Com. Japan) according to previous research (Xiao et al., 2010).

2.8. Statistical method and analysis software

The data were analyzed using SPSS statistical software (V 11.5, Chicago, IL, USA). Since not all treatments have the same sample size and alpha errors of the tests have only indicator value due to the multiple test problem, The significances of difference between various treatment groups of the same strain or mixed culture were analyzed with a one-way ANOVA followed by LSD multiple-comparison tests (e.g. in Figs. 3, Figs. 7 and 8), and it is

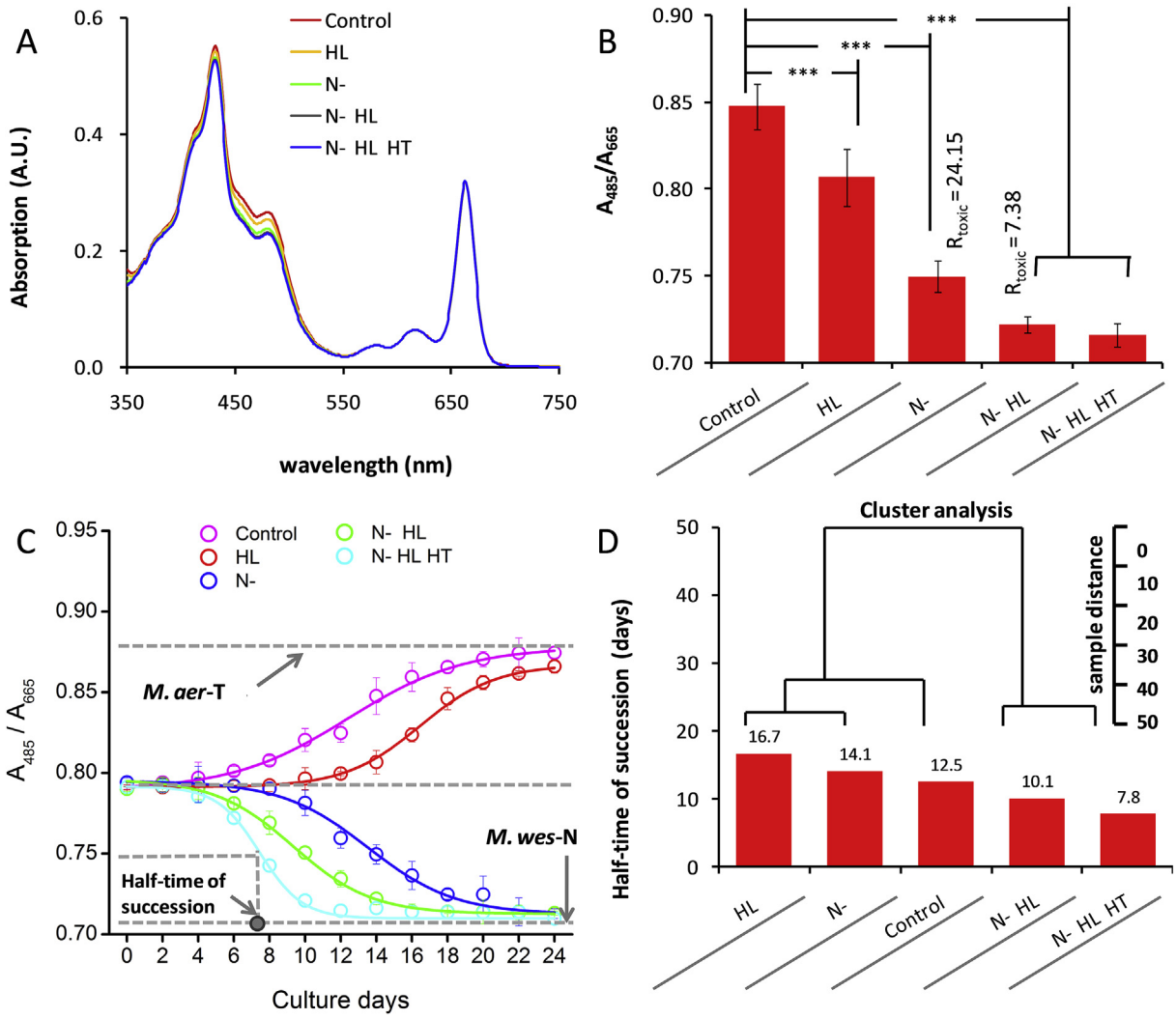

Fig. 7. The competition between toxic *Microcystis* (*M. aer*-T) and non-toxic *Microcystis* (*M. wes*-N) under various experimental conditions including five treatmented samples list as following: control, high light (HL), nitrogen limitation (N-), N- and HL (N- HL), N- HL and High temperature (N- HL HT). (A) Absorption spectrum of mixed cultures; (B) The ratio of *M. aer*-T and *M. wes*-N suggested by A_{485}/A_{665} on the 14th day during experimental period; (C) The population competition processes of *M. aer*-T against to *M. wes*-N indicated by A_{485}/A_{665}; (D) analysis of half-time of succession, which suggested that half of one strain was replaced by another strain. The level of significance of differences between these various treatments are indicated by *, **, *** for $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Table 2
The culture conditions of photosynthetic oxygen evolution experiments.

Culture conditions	Variable 1	Variable 2	Interactive effect of variables	Results shown in
Condition 1	T = 25 °C	P = 1.0 mg L^{-1}	$INT_{(L)} \times INT_{(N)}$	Fig. 4A–C
Condition 2	T = 25 °C	N = 30 mg L^{-1}	$INT_{(L)} \times INT_{(P)}$	Fig. 4D–F
Condition 3	P = 1.0 mg L^{-1}	L = 60 μmol photons m^{-2} s^{-1}	$INT_{(T)} \times INT_{(N)}$	Fig. 5A–C
Condition 4	N = 30 mg L^{-1}	L = 60 μmol photons m^{-2} s^{-1}	$INT_{(T)} \times INT_{(P)}$	Fig. 5D–F
Condition 5	N = 30 mg L^{-1}	P = 1.0 mg L^{-1}	$INT_{(L)} \times INT_{(T)}$	Supplement 1

Notes: INT, Interactive effect of variables;
$INT_{(L)}$ = (0, 11, 36, 62, 90, 137, 217, 416, 933 and 1382) μmol photons m^{-2} s^{-1};
$INT_{(N)}$ = (0, 0.1, 0.2, 0.4, 0.7, 1, 2, 4, 10 and 30) mg L^{-1};
$INT_{(P)}$ = (0, 0.01, 0.02, 0.04, 0.07, 0.1, 0.2, 0.4, 0.7 and 1) mg L^{-1};
$INT_{(T)}$ = (17, 19, 21, 23, 25, 27, 29, 31, 33 and 35) °C.

considered significant if *p*-value is 0.05 or less for a given *F* statistic test value. The significance difference of photosynthesis between any two strains was analyzed by *t*-test of independent samples (e.g. in Fig. 1 and Table 4), and it is considered significant if the probability of assuming no difference between two independent samples is less than 0.05 ($p < 0.05$). Relations between photosynthesis (or MCYST cellular quota or the ratio of toxic *Microcystis*) and various growth conditions such as light intensity, nutrient concentration, and temperature were fitted by exponential equation, and the significant levels between any two strains were also analyzed by *t*-test of paired samples (e.g. in Fig. 2). The distance method in SPSS software was used to analyze and cluster the difference of various

Table 3
Polymerase chain reaction primer sets used in this study.

Target	Primer	Sequence (5′–3′)	Target size	Reference
Microcystis 16S rRNA	MIC 184 F	GCC GCR AGG TGA AAM CTA A	230 bp	Neilan et al. (1997)
	MIC 431 R	AAT CCA AAR ACC TTC CTC CC		Rinta-Kanto et al. (2005)
Microcystis mcyD	*mcyD*-F	GGT TCG CCT GGT CAA AGT AA	298 bp	Baxa et al. (2010);
	mcyD-R	CCT CGC TAA AGA AGG GTT GA		Li et al. (2014)

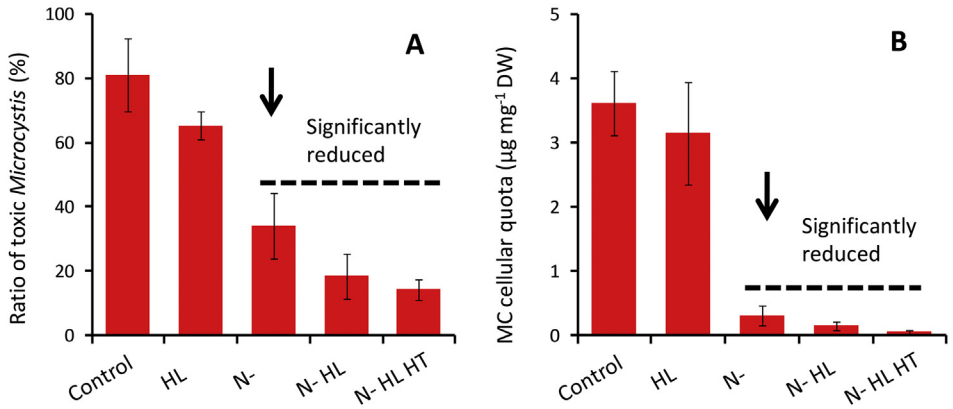

Fig. 8. The competition between toxic *Microcystis* (*M. aer*-T) and non-toxic *Microcystis* (*M. wes*-N and *M. aer*-N) under various experimental conditions including five treatmented samples list as following: control, high light (HL), nitrogen limitation (N-), N- and HL (N- HL), N- HL and High temperature (N- HL HT). (A) The ratio of toxic *Microcystis* calculated with real-time PCR method; (B) the MC quotas of *Microcystis* biomass (mg g^{-1} dry weight).

treatments including high light (HL), N-limitation (N-), HL & N-, HL & N- & high temperature, and control experiment, which was shown in Fig. 7D.

3. Results

3.1. Effects of light, nutrients and temperature on the photosynthesis of toxic and non-toxic Microcystis

When nitrogen concentration was as low as 0.1 mg L^{-1}, the photosynthesis of the toxic *M. aer*-T was the lowest among the three strains of *Microcystis* ($P_m = 6.487$ μmol O_2 (mg^{-1}chla) min^{-1}), which was only 45.7% of that cultured under N-rich condition (Fig. 1A, Table 4). Under N-limitation condition, the quantum efficiency of *M. aer*-T was seriously limited, and the half-saturated light (E_k) also decreased to a relatively low level (Table 4). All these results suggested that the limiting factor of the photosynthesis of *M. aer*-T was nitrogen rather than phosphorus. The photosynthesis of the non-toxic *Microcystis M. aer*-N was very low under P-limitation and low temperature conditions, which were 1.439 and 0.894 μmol O_2 (mg chla)$^{-1}$ min^{-1}, respectively, suggesting that the growth of *M. aer*-N might be seriously limited by P-deficiency and low temperature stress (Fig. 1C).

When cultured at 27 °C, the photosynthetic rate of *M. aer*-T was 7.8% and 109.1% higher than that of *M. wes*-N and *M. aer*-N, respectively (Table 4), and it was respectively 31.0% and 59.2% significantly higher than that of *M. wes*-N and *M. aer*-N when the temperature rose to 33 °C (the probability of assuming no difference between two independent samples, $p < 0.001$, *t*-test). This indicated that elevated temperature can significantly promote the competitive advantage of toxic *Microcystis* under suitable light and nutrients conditions. Half-saturated light (E_k) of *M. aer*-N measured at low temperature was significantly lower than that of other treatments, and it rapidly increased with the rise of temperature as well as the angle value of P-I curve (θ). Under N-limitation conditions, the E_k of *M. aer*-T was slightly lower than that of *M. wes*-N and *M. aer*-N (Table 4), suggesting that high-light tolerance of toxic *Microcystis* was lower than that of non-toxic species.

Cultured under dark or low light conditions, the photosynthesis of *M. aer*-N was slightly higher than that of *M. aer*-T, which might be attributed to a relatively lower respiration rate. The photosynthesis of toxic *Microcystis* (*M. aer*-T) was always significantly higher than that of non-toxic strains (*M. wes*-N and *M. aer*-N) (both of the significant levels were $p < 0.001$) (Fig. 2A). Fig. 2B showed that *M. wes*-N was the winner when nitrogen was limited while *M. aer*-T would be the winner when nitrogen was sufficient. The effects of phosphorus on the competition between *M. aer*-T and *M. wes*-N were similar with that of nitrogen (Fig. 2C). Under saturated light, photosynthesis of *M. aer*-N was always the lowest in the three strains while *M. aer*-T was always the highest, and the photosynthetic advantage of *M. aer*-T slightly increased with the rise of temperature (Fig. 2D).

3.2. Changes of quantum efficiency with light, nutrients and temperature

The inhibition ratios of photosynthetic quantum efficiency (IRPQ) of *M. aer*-T under N-limitation and low-temperature stress were 62.6% and 74.2%, respectively. The quantum efficiency of *M. aer*-N was significantly suppressed by N-limitation, P-limitation and low-temperature stress ($p < 0.001$), especially IRPQ under P-limitation condition was 99.1% (Fig. 3A and C). IRPQ of *M. wes*-N was higher than 60% only under low temperature (Fig. 3C). The quantum efficiency of *M. aer*-T was significantly higher than that of *M. wes*-N and *M. aer*-N when exposed to saturated light (Fig. 3B). In various concentrations of nitrogen and phosphorus, the quantum efficiencies of *M. aer*-N were always significantly lower than those of *M. aer*-T and *M. wes*-N ($p < 0.001$) while the quantum efficiency of *M. aer*-T was significantly higher than that of *M. wes*-N (Fig. 3D). The quantum efficiency of each strains rose linearly with the increase of temperature and *M. aer*-T always had the highest values while *M. aer*-N had the lowest values (Fig. 3E).

Table 4
The parameters of photosynthetic light-response curves (P-I curves) of *Microcystis* strains when cultured under various conditions.

Strains used in experiment	Culture conditions	P_{max} [μmol O_2 (mg chla)$^{-1}$ min^{-1}]	R_{day} [μmol O_2 (mg chla)$^{-1}$ min^{-1}]	Θ [A.U.]	Φ [μmol O_2 (mg chla)$^{-1}$ (μmol photons)$^{-1}$]	E_k [μmol photons m^{-2} s^{-1}]	R^2
Toxic *Microcystis aeruginosa* (*M. aer*-T)	N = 0.1	6.487	1.287	0.946	0.0696	70.018	0.993
	N = 1.0	9.134	1.086	0.763	0.0896	83.674	0.992
	N = 10	14.202	0.739	0.927	0.0928	108.249	0.998
	P = 0.01	10.039	1.103	0.840	0.0617	83.789	0.998
	P = 0.1	12.440	1.092	0.944	0.0854	99.590	0.996
	P = 0.7	14.927	1.020	0.887	0.0922	107.144	0.998
	T = 19 °C	8.979	0.982	0.894	0.0518	96.537	0.995
	T = 27 °C	23.364	0.353	0.643	0.1053	114.853	0.988
	T = 33 °C	44.029	0.973	0.522	0.1349	178.919	0.990
Non-toxic *Microcystis wesenbergii* (*M.wes*-N)	N = 0.1	10.179	1.192	0.888	0.0623	89.296	0.996
	N = 1.0	13.720	1.606	0.752	0.0738	102.743	0.998
	N = 10	15.454	1.343	0.569	0.0732	113.286	0.998
	P = 0.01	13.478	1.392	0.100	0.0528	139.288	0.997
	P = 0.1	18.251	1.346	0.395	0.0760	129.308	0.997
	P = 0.7	18.784	1.388	0.172	0.0675	147.448	0.999
	T = 19 °C	7.487	0.959	0.366	0.0364	111.025	0.995
	T = 27 °C	21.673	1.492	0.362	0.0871	137.658	0.993
	T = 33 °C	33.604	3.357	0.515	0.1173	175.678	0.992
Non-toxic *Microcystis aeruginosa* (*M. aer*-N)	N = 0.1	3.510	1.083	0.651	0.0199	88.609	0.971
	N = 1.0	4.579	0.508	0.980	0.0333	72.926	0.905
	N = 10	7.161	0.405	0.841	0.0355	101.984	0.978
	P = 0.01	1.439	0.761	0.980	0.0152	49.903	0.333
	P = 0.1	5.546	0.336	0.980	0.0311	97.585	0.868
	P = 0.7	7.228	0.277	0.935	0.0334	114.146	0.952
	T = 19 °C	0.894	0.365	0.980	0.0154	14.325	0.182
	T = 27 °C	11.175	0.658	0.812	0.0529	113.473	0.961
	T = 33 °C	27.664	2.389	0.100	0.0807	194.824	0.997

Note: P_{max}, the maximum photosynthetic efficiency; R_{day}, respiration rate under light condition; θ, the angle value of non-linear fitting equation; φ, the apparent quantum efficiency; E_k, half-saturation point of light intensity for photosynthesis; AU, arbitrary unit.

3.3. Photosynthetic comparison between toxic and non-toxic *Microcystis* under various co-regulated conditions

There was no significant difference in photosynthetic rate between *M. aer*-T and *M. wes*-N under near dark condition. With the increase of light, *M. aer*-T gradually became dominant species. Under high light conditions, N-rich medium would result in a greater competitive advantage of *M. aer*-T than that of *M. wes*-N, but this advantage could be reversed by N-limitation (Fig. 4A). The co-regulated effects of phosphorus and light on photosynthetic competition were similar with that of nitrogen and light, except the competitive advantage of *M. wes*-N reached the peak when phosphorus was at a moderate level other than at lowest level (Fig. 4D). The photosynthetic rate of *M. aer*-T was always higher than that of *M. aer*-N except low light stress, and this photosynthetic advantage would become greater and greater with the increase of light intensity as well as nitrogen or phosphorus concentration (Fig. 4B and E). The photosynthetic competition between toxic *Microcystis* and non-toxic *Microcystis* was mainly regulated by nitrogen other than light, and N-deficiency combined supersaturated light could significantly enhance the dominance of toxic *Microcystis* (Fig. 4C). Under various phosphorus concentrations, the photosynthetic rate of toxic *Microcystis* was always higher than that of non-toxic *Microcystis* and it reached the lowest value at a moderate phosphorus level (Fig. 4F).

Non-toxic *Microcystis* had a greater competitive advantage than toxic species under N-limitation combined with high temperature conditions (Fig. 5A). *M. aer*-T was a winner in the competition with *M. wes*-N under P-limitation condition while it became a loser when phosphorus is sufficient (Fig. 5D). The photosynthetic rate of *M. aer*-T was always higher than that of *M. aer*-N, which was more significant with the increase of temperature and nutrients (Fig. 5B, E). Considering the competition among the three strains, the competition between toxic and non-toxic *Microcystis* was primarily determined by nitrogen other than temperature, and the competitive advantage of toxic *Microcystis* greatly reduced under high temperature combined with N-limitation (Fig. 5C). Cultured at various temperature and phosphorus concentrations, the photosynthetic rate of toxic *Microcystis* was always higher than that of non-toxic strains (Fig. 5F).

3.4. Weight analysis of photosynthesis for light, nutrient and temperature

Nitrogen rather than light had a greater effect on the photosynthesis of *M. aer*-T and *M. aer*-N while light had a greater weight on the photosynthesis of *M. wes*-N than other factors did (Fig. 6A–C). The weight analysis of phosphorus on the photosynthesis of the three *Microcystis* strains was similar with that of nitrogen (Fig. 6D–F). In the comparison analysis of weight between nutrient and temperature, the photosynthesis of *M. aer*-T and *M. aer*-N was mainly affected by nutrients (N and P) rather than temperature while it was mainly affected by temperature for *M. wes*-N (Fig. 6G-L).

3.5. Population competition between toxic and non toxic *Microcystis* analyzed by absorption spectrum

There were some differences in the absorption peaks of 685 nm after the normalization of all the spectrum scanning curves (Fig. 7A). The A_{485}/A_{665} of mixed culture significantly decreased by 4.8% under high light condition while it significantly decreased by 11.5% when suffering the single N-limitation. The co-regulated effect of N-limitation and high light would cause toxic strain significantly ($p < 0.001$) decreased to 7.38% (i.e., $R_{Toxic} = 7.38\%$) (Fig. 7B). The A_{485}/A_{665} of various treatments almost reached to a stable value on the 18th day of the growth period, and the A_{485}/A_{665} in mixed cultures with various N-limitation treatments had no

differences with that of *M. aer*-T while had significant differences with that cultured under N-rich conditions (Fig. 7C). The cluster analysis suggested that the half-time of succession between toxic and non-toxic *Microcystis* under co-regulated effects of high light and N-limitation was significantly shorter than that of single N-limitation treatment (Fig. 7D).

3.6. Regulation of various N-limitation treatments on the MCYST cellular quota and ratio of toxic Microcystis analyzed by qPCR

When grew in N-rich treatments, the ratio of toxic *Microcystis* gradually increased, and it reached to 81.2% and 65.3% on the 18th dayunder control and high-light conditons, respectively (Fig. 8A). But the percentages of toxic *Microcystis* in mixed culture gradually lowered to 34.0% at the end of experiment when only suffered for N-limitation, which would further lowered to 18.4% under the co-regulations of N-limitation and high light stress, and this interactive effects would be amplified by rising temperature. The MCYST cellular quotas in mixed culture were significantly decreased to 8.6% under N-limitation condition in comparison with that of control, and this decline ratio was lower than the percentages decrease of toxic *Microcystis* (Fig. 8B), which suggested that MCYST-production process would be inhibited by N-limitation treatment. Also, increase of light intensity and temperature could cause further decrease of the MCYST cellular quotas in mixed culture.

4. Discussions

4.1. N-rich or low light favors the competitive advantage of toxic Microcystis

In order to reveal the regulations of environmental factors such as light intensity, nutrients and temperature on the competition of *Microcystis*, photosynthesis of three strains of *Microcystis* was measured and compared. Under various concentrations of nitrogen and phosphorus, both of the photosynthesis and the quantum efficiencies (shown in Figss. 1–3) of *M. aer*-N were always significantly lower than that of *M. aer*-T and *M. wes*-N (the probability of assuming no difference between two independent samples, $p < 0.001$, *t*-test). The photosynthesis of naturally mutated non-toxic *Microcystis* is relatively low, which may be related with the physiological role of MCYST in the processes of growth and stress resistance. Therefore, when we analyze the co-regulation effects of various factors on the competition between toxic and non-toxic *Microcystis*, we should pay more attention to the competition between *M. aer*-T and *M. wes*-N.

Due to the specific affinity of different strains to nutrients, the fluctuation of nutrients might have a significant impact on the interspecific competition and toxicity of *Microcysits* bloom. The results of Figs. 4 and 5 suggested that nitrogen rather than phosphorus determined the competition between toxic *Microcystis* and non toxic *Microcystis*, which was consistent with previous studies that reported the nitrogen availability was a very common limiting factor in controlling summer and autumn algal blooms and their toxicity (Xu et al., 2015; Horst et al., 2014). We found N-limitation alone could not determine whether toxic *Microcystis* became a winner of competition, and the competitive advantage of toxic *Microcystis* caused by N-limitation only presented under low or moderate light conditions. This indicates that a co-regulated effect of N-limitation and light limitation is beneficial to the dominance of toxic *Microcystis*, and light intensity play an important role in the process of competition.

Low light restricted the photosynthesis and growth rate of *Microcystis*, and the photosynthesis of toxic strain was slightly higher than that of non-toxic strains. The weight of light was higher than that of nitrogen when *Microcystis* was subjected to low light stress combined with N-limitation, which suggested light utilization efficiency (i.e. quantum efficiency) would determine that toxic *Microcystis* was the winner when cultured under N-deficiency condition. The quantum efficiency of toxic *Microcystis* was higher than that of non-toxic strains, which suggested that toxic *Microcystis* would have more competitive advantage in comparison with non-toxic species under low light condition. Theoretically, under low light condition, most of absorbed light energy will be used for the normal growth of algae instead of the synthesis of seemingly unnecessary secondary metabolites such as MCYST. And production of MCYST is usually an extra burden of normal growth (Briand et al., 2012). Indeed, the MCYST synthetase genes involved in peptide synthesis were downregulated under N deprivation, however, N-limitation simultaneously led to the upregulation of MCYST synthetase genes involved in tailoring and transportation (Harke and Gobler, 2015). It has been reported that the NtcA gene binding to the region of *mcy* operon increased under N-limiting conditions, which suggested that *mcy* transcription and MCYST production was enhanced by N-limitation (Kuniyoshi et al., 2013). And perhaps, *Microcystis* can promote its own growth rate by inducing an increase in MCYST cellular quotas, especially under N-limitation conditions (Long et al., 2001) or light-limited conditions (Wiedner et al., 2003).

Therefore, under growth-limiting conditions, the benefits of MCYST production outweigh the costs (Briand et al., 2008), and the competitive advantage of toxic *Microcystis* possibly be related to the synthesis process of MCYST. This speculation is supported by the fact that ATP content in algal cells would sharply decrease due to a significant down-regulation of photosynthetic genes when MCYST synthetase gene *mcyB* was knocked out (Makower et al., 2015). Therefore, MCYST-producing *Microcystis* may have a relatively high nitrogen uptake rate due to sufficient ATP, especially under N-limitation and light-limitation conditions. All these speculations were have been further confirmed that the quantum efficiency of toxic *Microcystis* was significantly improved under N-limitation (as shown in Table 4 and Fig. 3D). And Fig. 4A suggested that the low light range within which toxic *Microcystis* would have competitive advantage was below 36 μmol photons m^{-2} s^{-1} companied with N-limitation.

When cultured at low light intensity, we found the competitive advantage of toxic *Microcystis* was slightly enhanced with the increase of N concentration, which suggested that the N-availability would increase the toxicity of *Microcystis* blooms. Also, previous researches have shown that N addition could significantly promote the growth and toxicity of cyanobacterial blooms in lakes (Davis et al., 2015), and this increased toxins might be caused by the increasing weight of light (as shown in Fig. 6). The weight of light was higher than that of nitrogen even though under low-light and N-limitation conditions, and it would be greater with the increase of N concentration. Therefore, the competitive advantage of toxic *Microcystis* still existed and even slightly more significant due to a higher light utilization efficiency (i.e. quantum efficiency) than that of non-toxic species. The promotion of N increase to the growth of toxic *Microcystis* cultured at low light level might be explained by the relieved growth inhibition of extra burden relating with MCYST production (Briand et al., 2012) as well as the enhanced promotion effect of MCYST on the toxic cyanobacteria suffering for environmental stress (Briand et al., 2008).

It has been reported that a higher MCYST cellular quota was acquired when *Microcystis* or *Planktothrix* were exposed to higher light under the conditions of adequate nutrients and light intensity below saturated light (Wiedner et al., 2003; Tonk et al., 2005; Sevilla et al., 2012; Dai et al., 2016), and toxic *Microcystis* is more tolerant to high light stress than non-toxic *Microcystis* (Phelan and

Downing, 2011), which might be attributed to the stronger resistance to oxidative stress existing in toxic *Microcystis* than that in non-toxic strains (Dziallas and Grossart, 2011; Zilliges et al., 2011). The comparison between toxic *Microcystis* and MCYST-lacking mutant of *Microcystis* suggested that the transcriptional level of MCYST-related protein strongly reduced in MCYST-lacking mutant under all light conditions (Dittmann et al., 2001), so MCYST production induced by redox state of the photosynthetic apparatus (Deblois and Juneau, 2010) might contribute to the formation of some important proteins relating to resistance against high-light stress. In this study, we found that under N-rich conditions, the photosynthetic competitive advantage of MCYST-producing *Microcystis* against non-toxic species was significantly enhanced by the increase of light intensity, which might be attributed to the potential photoprotective function of MCYST through partly bounding to proteins such as the lectin MVN, the glycoprotein MrpC, phycobilisomes (PBSs) and the large subunit of Rubisco (RbcL) (Zilliges et al., 2011; Kaplan et al., 2012). When cultured at high light, MCYST cellular quota decreased under nitrogen limitation and it increased significantly under nitrogen sufficient conditions (Horst et al., 2014), which indicated that the co-regulated effect of light and N-availability was beneficial to the increase of MCYST cellular quota. These increased MCYST could enhance the structural stability and functional activity of the photosynthetic proteins or enzymes by binding to the sulfhydryl group (-SH) (Kaplan et al., 2012), thus enhancing the adaptability of algae to high light stress. Also, comparison between wild-type strain and its *mcyB* mutant indicated that MCYST was involved in intracellular processes instead of growth regulation (Hesse et al., 2001), which might enhance the adaptability to the deficiency of intracellular inorganic carbon (Jähnichen et al., 2007), or increase the resistance to oxygen free radicals (Dziallas and Grossart, 2011). Therefore, all these researches suggest MCYST have many important physiological functions, which favor the competitive advantage of MCYST-producing strains. So far, various function of MCYST have been proposed and evidenced in many studies, but its specific mechanisms needs further study.

4.2. Combination of N-limitation and high light promotes the competitive advantage of non-toxic Microcystis and reduces the MCYST cellular quota of Microcystis bloom

Under the condition of nitrogen deficiency, with the increase of light intensity, the determinants of competition between toxic and non toxic *Microcystis* gradually changed from light limitation to nutrient limitation. At room temperature and sufficient illumination light (Fig. 3C), the photosynthesis of toxic *Microcystis* was significantly inhibited by N-deficiency while that of non-toxic *Microcystis* was suppressed by P-deficiency (i.g. *M. aer*-N) or not affected by nutrients (i.g. *M. wes*-N), which indicated that N-deficiency would significantly reduce the competitive advantage of toxic *Microcystis*. The same results were shown in Figs. 7B and 8A, which suggested that N-deficiency is a key factor in determining the competitive advantage of toxic *Microcystis* when sufficient light is available. Also, the co-regulated impacts of light and nitrogen on photosynthetic competition were further confirmed (Fig. 4A). These results are consistent with previous research which suggested that the availability of N in summer is a key growth-limiting factor for toxic *Microcystis* blooms (Xu et al., 2010, 2015). Under the same N-limitation conditions, low light will lead to the competitive advantage of toxic *Microcystis*, while high light will cause the non-toxic *Microcystis* the winner of competition. This may be because N-limitation mainly restricts the transmembrane transport of nutrients under low light conditions, while it principally suppresses the recovery of photosynthetic apparatus damaged by high light.

When exposed to supersaturated light, the reaction centers of photosynthetic systems may be excessively excited by receive of mass energy, leading to further results in the formation of reactive oxygen species (ROS) which are lethal to photosynthetic organisms (Asada, 2006). To survive, photosynthetic organisms have evolved several adaptation strategies including three main pathways: (1) the formation of excitation energy traps which refer to the synthesis of light-induced proteins and/or conformational changes of pigment proteins (Wilson et al., 2007; Wang et al., 2012b); (2) rapid recovery of damaged D1 protein in photosystem II (PSII) (Prasil et al., 1992); (3) up-regulation of antioxidant enzyme activities (Gill and Tuteja, 2010). All three processes involve with the regulation of protein synthesis and consume large amounts of ATP and nitrogen. So, under N-limitation conditions, MCYST production of toxic *Microcystis* may suppress its own growth due to nitrogen competition with above mentioned protein synthesis processes. Based on these analyses, in theory, N-limitation will cause MCYST-producing *Microcystis* be less tolerant to high light stress and be more susceptible to photo-inhibition, which may be related to the decrease of MCYST cellular quota resulted from N-limitation (Horst et al., 2014) and the photoprotection function of MCYST (Kaplan et al., 2012). This deduction was validated by a lower half-saturated light intensity when toxic *Microcystis* was cultured under N-limitation conditions (Table 4). Therefore, increasing light combined with N-limitation will significantly reduce the competitive advantage of MCYST-producing *Microcystis*. It is also possible that N-limitation inhibits the production of MCYST, which makes the photoprotection of pigments in non-toxic *M. wesenbergii* more acute than that of toxic *M. aeruginosa*.

To a certain extent, population dynamics of toxic *Microcystis* determines the MCYST cellular quota. So, laboratory experiments of mixed culture were carried out to further investigate the effects of N-limitation combined with other factors on the MCYST production of *Microcystis*. The results showed that N-limitation combined with moderate light significantly decreased the ratio of toxic *Microcystis* to non-toxic species, and the decrease degree and decrease rate could be significantly aggravated by increasing light intensity (Figs. 7 and 8). Nitrogen addition could result in a rapid increase of bloom biomass and MCYST concentration in Lake Erie (Davis et al., 2015), which also suggested that N-limitation could suppress the competitive advantage of toxic *Microcystis*. Horst et al. (2014) reported that the N-limitation lowered the MCYST cellular quota, but up-regulated *mcyA* or *mcyB* genes (Yoshida et al., 2007; Horst et al., 2014). This suggested that N availability not only affected the expression of MCYST genes, but also regulated the ratio of toxic *Microcystis* to non-toxic *Microcystis*. N-limitation significantly reduced the dominance of toxic *Microcystis* and its MCYST production, which would subsequently result in a very low level of MCYST cellular quota. However, thedecrease of MCYST production could be significantly enhanced by the co-regulated effects of increasing light.

4.3. Temperature rise amplifies the competition between toxic and non-toxic Microcystis

The nutrients uptake and photosynthesis of algae are significantly affected by temperature, which is the most important factor determining the photosynthetic rate and succession direction of toxic cyanobacteria (Davis et al., 2009; Wang et al., 2016). Dziallas and Grossart (2011) proposed that global warming would significantly increase the toxicity and potential toxicity of cyanobacterial blooms, which was explained by temperature-driven changes in associated bacterial communities. Our results are consistent with all these reports, that is, temperature is the main limiting factor for

the growth of *Microcystis* when cultured under N-limitation and low temperature stress conditions. Low temperature can seriously restrict the fluidity of thylakoid membranes, which subsequently results in a significant decrease of electron transport rate in PSII, and even leads to photoinhibition and photodamage (Wang et al., 2010, 2016). As mentioned above, the defense against photoinhibition should be associated with the synthesis of many light-adaptive proteins and will deplete a large amount of nitrogen resource, which will lead to a relative lower photosynthetic rate of toxic *Microcystis* than that of non-toxic species when growing under N-limitation and low temperature conditions (Fig. 5A). This competitive disadvantage of toxic *Microcystis* was significantly different from the results shown in Fig. 4A, which might be attributed to the serious limitations of MCYST-production and/or associating nitrogen transport by low temperature. When nitrogen and phosphorus were sufficient, the photosynthetic rate of toxic *Microcystis* always had higher photosynthesis than that of non-toxic strains, and this competitive advantage was gradually enhanced with the increase of temperature and light intensity (Supplement 1).

It is necessary to analyze the co-regulated effects of light and temperature on the photosynthesis of the three strains. The results showed that temperature generally had a higher weight than light, and its impact on *M. aer*-N seemed to be significantly greater than that of *M. aer*-T and *M. wes*-N, which suggested that low temperature stress will reduce the competitive advantage of *M. aer*-N and make the succession between *M. aer*-T and *M. wes*-N more important (Supplement 2).

When light was sufficient for photosynthesis, the weight of nutrient limitation gradually increased and became the key factor affecting the competition between toxic and non-toxic *Microcystis*. The photosynthetic rate and growth rate were significantly promoted by temperature rise, which further aggravated the inhibition of N-limitation on the growth of toxic *Microcystis*. This deduction can be initially confirmed by the fact that a lower enhancing effect of higher temperature on the photosynthesis of toxic *Microcystis* than that of non-toxic *Microcystis* (Fig. 3A). Temperature rise can increase the toxic proportion and intracellular MCYST content of *Microcystis* blooms (Joung et al., 2011), which will further amplify the limitation impacts of N-deficiency on the photosynthesis of toxic *Microcystis* as (Fig. 5A).

4.4. Implications of this study for the control of toxic Microcystis blooms in eutrophic lakes

The results of this work have potential implications for understanding the toxicity control of *Microcystis* blooms. Although *Microcystis* biomass was effectively decreased by reducing nutrient input (Paerl and Otten, 2013), the ultimate guarantee of water quality safety should be the control of toxic *Microcystis* biomass and MCYST. Previous study has suggested that MCYST production was regulated by multiple environmental factors rather than a single factor (Jähnichen et al., 2011). We found that nitrogen rather than phosphorus or temperature was the key factor affecting the competition between toxic *Microcystis* and non-toxic species when sufficient light was available (Fig. 5C and F), and N-limitation combined with high light could significantly reduce the competitive advantage of toxic *Microcystis* (Fig. 4C and F). Based on these results, we can speculate that the succession between toxic and non-toxic *Microcystis* in lakes depends on whether nitrogen is a limiting factor when light is saturated in summer. The biomass of *Microcystis* was distributed vertically in the water column, and the *Microcystis* in lower layer would suffer serious light limitation (Wang et al., 2012c). According to above analysis, this low light accompanied by nitrogen-rich conditions can well explain why toxic *Microcystis* dominates in the early stage of bloom formation. With the rapid increase of *Microcystis* biomass, nitrogen gradually became the limiting factor for algal growth. In this case, with the continuous increase of sunlight intensity in summer, the initial dominance of toxic *Microcystis* will be replaced by non-toxic strains, and this succession process may be promoted by temperature rise in late summer.

MCYST concentration in water column is not only related to the biomass of *Microcystis* but also to the MCYST cellular quota (Jähnichen et al., 2001; Horst et al., 2014). Therefore, to effectively control the toxic *Microcystis* blooms in eutrophic lakes, we should try to reduce the total biomass of *Microcystis* and the competitive advantage of toxic *Microcystis*. Based on the co-regulated effects of light, nutrients and temperature, it is necessary to control nutrients, especially by intercepting nitrogen pollutants and increasing the light intensity and transparency in water column via restoring submerged plants. Moreover, once water transparency is improved, the activity of photosynthetic bacteria will be enhanced (Wang et al., 2014) and most of the nitrogen will be removed from water and sediments, which will further enhance the effect of nitrogen control on the reduction of MCYST concentration in eutrophic lakes.

5. Conclusions

To reveal the regulation mechanism of various factors on the population competition between toxic and non-toxic *Microcystis*, we measured algal photosynthesis and found that light and nutrient were the key factors for their competition. Both photosynthesis and quantum efficiency suggested that toxic *Microcystis* rather than non-toxic *Microcystis* was seriously restricted by N-limitation. Weight analysis indicated that nitrogen account for a greater influence in toxic *Microcystis* than that of non-toxic species when cultured under high light or high temperature. The comparison of photosynthetic characteristics showed that the combination of low light and N-limitation enhanced the competitive advantage of toxic *Microcystis*, while the combination of high light and N-limitation enhanced the competitive advantage of non-toxic *Microcystis*, and these two competition advantages were further amplified as temperature increased. Mixed competitive experiments of toxic and non-toxic *Microcystis* indicated that co-regulated effects of N-limitation and high light could significantly reduce the ratio of toxic *Microcystis* to non-toxicstrains, which also lead to a significant decrease of MCYST cellular quota in *Microcystis* biomass.

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Appendix A. Supplementary data

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