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ORIGINAL ARTICLE

Photosynthetic response to nitrogen source and different ratios of nitrogen and phosphorus in toxic cyanobacteria, *Microcystis aeruginosa* FACHB-905

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ABSTRACT

The frequent outbreak of cyanobacterial blooms has become a worldwide phenomenon in freshwater ecosystems. Studies have elucidated the close relationship between harmful algal blooms and nutrient contents, including the loading of nitrogen and the ratios of nitrogen (N) and phosphorus (P). In this study, the effect of inorganic (nitrate and ammonium) and organic (urea) nitrogen at varied N/P ratios on the Microcystis aeruginosa *FACHB-905 accumulation and photosynthesis was investigated. The optimal NO_3/P in this study were 30~50 indicated by the cell abundance (4.1×10^6/mL), pigment concentration (chlorophyll a 3.1 mg/L, phycocyanin 8.3mg/L), and chlorophyll fluorescence parameters (*rETR, E_k, α, φPSII *and* F_v/F_m *values), while too high NO_3-N (N/P=100:1) would cause an intracellular nitrate inhibition, leading to a decrease of photosynthetic activity. In addition, low concentration of NH_4-N (N/P=4:1) would favor the* M. aeruginosa *growth and photosynthesis, and high NH_4/P ratio (>16) would rise the ammonium toxicity of algal cells and affect the N assimilation. In urea treatments,* M. aeruginosa *responded similarly to the NH_4-N treatments both in growth curves and pigment contents, and the favorable N/P ratio was between 16~30, suggested by the chlorophyll fluorescence parameters. The results demonstrated that the various chemical forms of N and N/P ratios have a significant impact on* Microcystis *abundance and photosynthesis. More work is needed to figure out the mechanism of nitrogen utilization by* Microcystis *and the photosynthetic response to nutrient stress at the molecular level.*

Key words: Microcystis*; nitrogen form; N/P ratio; photosynthesis; chlorophyll fluorescence parameters.*

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INTRODUCTION

Harmful algal blooms (HABs) have been a major environmental concern in both marine and freshwater throughout the world (Wilhelm *et al.,* 2014; Lehman *et al.,* 2015). Among the organisms involved, *Microcystis* is perhaps the most notorious, since the hepatotoxins (especially microcystins) produced can decrease the richness of wild animals (White *et al.,* 2005), poison human and domestic livestock (Peng *et al.,* 2015). The environmental parameters particularly temperature, light, high pH/low CO_2, nutrient availability and trace elements have been suggested to lead to cyanobacterial dominance (Barbiero *et al.,* 1999; Aneesh *et al.,* 2015).

Phosphorus is widely accepted as the main nutrient controlling the development of natural populations of cyanobacteria in many freshwater environments for decades (Schindler, 1977). However, the potential role of nitrogen concentrations and chemical forms in driving *Microcystis* bloom formation and biological community structure has gained widespread attention in recent years. It has been suggested that high NO_3-N level promotes the advantage of *Microcystis* (Lehman *et al.,* 2009). Opposite results were obtained by Moisander *et al.* (2009) who found that *Microcystis* abundance increased in response to all forms of N, at no limiting conditions. In addition, Donald *et al*. (2011) explored that the NH_4-N and urea favored non-heterocystous cyanobacteria, and microcystin production was increased by up to 13-fold after N added. Belisle *et al*. (2016) also evaluated the urea distribution and the urease activity in Lake Erie and confirmed that organic nitrogen source was an important driver of cyanobacterial blooms and toxin production.

Among the major hypotheses that have been proposed to explain the cyanobacteria bloom, the prevalent may be that of the TN/TP ratio. Smith (1983) put forward that the low N/P ratios (<29, by mass) favored dominance by blue-green algae in lake phytoplankton, which promoted the research of N/P ratio on the HABs. Hyenstrand *et al.* (1998) concluded that a low N/P mass ratio (29) was one of the nine main factors influencing the success of cyanobacteria, while high N/P mass ratios (20-50) lead to a community dominated by green algae (Bulgakov and Levich, 1999) or diatoms (Mccarthy *et al.,* 2009). Nevertheless, Nalewajko and Murphy (2001) found that the optimal growth rate of *Microcystis aeruginosa* isolated from the Lake Biwa occurred at the N/P mass ratio of 100:1 and declined at lower ratios (N limitation) and high ratios (P limitation).

Pulse Amplitude Modulated (PAM) fluorometry has become one of the most common, non-invasive and rapid

techniques to measure the variability of chlorophyll fluorescence to monitor the photosynthetic response of microalgae under different nutrient conditions (Wang *et al.,* 2010). The following PAM parameters are generally used to measure the physiological state of the organism: rapid light curves (RLCs), relative photosynthetic electron transport (*rETR*), light saturation (E_k), photosynthetic efficiency (α), actual photochemical efficiency of PSII ($\varphi PSII$) and maximum quantum photochemical efficiency of PSII (F_v/F_m). Specifically, RLCs provided detailed information on the saturation characteristics of the electron transport chain as well as the overall photosynthetic performance exhibited by the microalgae (Ralph and Gademann, 2005). *rETR* is a measure of the rate of linear electron transport through PSII, while E_k defines the onset of light saturation as obtained from the curves (Juneau *et al.,* 2005). $\varphi PSII$ and F_v/F_m are used to estimate nutrient limitation, which have been found to decrease in nutrient stressed cultures (Schreiber *et al.,* 2002; White *et al.,* 2011).

Despite the clear importance of nitrogen in aquatic systems, the effects of various chemical forms of N and N/P ratios on *Microcystis* abundance are not consistent in previous studies, and few have evaluated the response of *Microcystis* photosynthetic activity. The goal of this study was to address this knowledge gap, studying the response of *Microcystis* to different forms of N and at different N/P ratios. In our experiments, *Microcystis aeruginosa* were cultivated in three different conditions, using chemical forms of nitrogen and different mass ratios of N/P medium. Cell numbers, key pigment concentration and chlorophyll fluorescence parameters (*rETR,* E_k, α, $\varphi PSII$ *and* F_v/F_m) were measured to figure out the microalgal cell growth and photosynthetic response to nutrient treatments, which aims to determine if different chemical forms of N and N/P ratio influence the accumulation of *Microcystis* biomass and photosynthesis.

METHODS

Strain and experimental design

Microcystis aeruginosa (FACHB-905) was purchased from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China), cultured with BG-11 medium (Rippka *et al.,* 1979). Cultures in exponential phase were centrifuged at 6000 xg for 10 min, and the pelleted cells were washed three times with nitrogen-free BG_{11} medium. Subsequently, cultures were nitrogen starved for 72 h under the conditions of 25°C, light intensity of 40 μmol photons $m^{-2}\,s^{-1}$, and a 12:12 LD cycle. After nitrogen starvation, the cells were re-inoculated into modified BG11 medium, with the initial cell density of 2.16×10^6 mL^{-1}. Three chemical forms of nitrogen were chosen: nitrate ($NaNO_3$), ammonium (NH_4Cl) and urea ($[NH_2]_2CO$) at different mass ratios of N/P: 100:1, 50:1, 30:1, 16:1, 4:1. The phosphorus concentration in each treatment was set as 1.0 mg L^{-1} (as K_2HPO_4).

Measurement of cell abundance

The cell abundance was determined daily by measuring the absorbance at 650 nm of each treatment. The relationship between the absorbance and the algal cell abundance was studied previously, with a gradient concentration of *M. aeruginosa* solutions cultured with standard BG_{11} medium. The number of single cells was counted using a haemocytometer (QIUJING, Shanghai, cell depth: 0.100 mm ± 2% (1/10 mm), ruling pattern: improved Neubauer, 1/400 square mm) while the absorbance at 650 nm was measured with a spectrofluorophotometer (UV-2100, UNICO, Shanghai, China). The relationship between the absorbance and the cell density fits the linear regression equation:

$$y = 5.0856x - 0.2132$$

Where x is the absorbance at 650 nm, y is the algal cell density (10^6 mL^{-1}), $R^2 = 0.9717$.

Measurement of pigment concentration

To determine the pigment concentration, 50 mL samples were filtered onto Whatman GF/C filters (0.22 μm) every three days. Chlorophyll-*a* was extracted using 90% acetone for 24 h in the dark at 4°C (Parsons *et al.,* 1984). For phycocyanin (PC), the filters were extracted using 0.05M (pH6.8) phosphate buffer solution (PBS) and then processed by repetitive freezing in liquid nitrogen and thawing at 4°C for three times (Padgett and Krogmann, 1987). The absorbance at 630, 645, 663 and 750 nm were measured to calculate the concentration of chlorophyll-*a* according to the formula (Parsons *et al.,* 1984):

$$C(mg\,L^{-1}) = 10^{-3}[11.64\,(0D_{663}-0D_{750}) - 2.16(0D_{645}-0D_{750}) + 0.10(0D_{630}-0D_{750})]V_1\,V^{-1}\,\delta^{-1}$$

where:
V is the volume of the sampling (50 mL);
V_1 is the constant volume of the extracted cultures each time (10 mL);
δ is the range of the cuvette (1 cm).

As to the phycocyanin, the concentration was calculated from the following equation (Padgett and Krogmann, 1987):

$$C(mg\,L^{-1}) = 10^3\,(0D_{620} - 0.7\,0D_{650})\,7.38^{-1}$$

Photochemical activity assay

Differences between the N forms and N/P ratio treatments were investigated with a WATER-PAM Chlorophyll Fluorometer (Walz, Effeltrich, Germany). The

Rapid Light Curves (RLC) were measured by increasing the actinic irradiance stepwise from 56 to 915 μmol photons m^{-2} s^{-1} with a saturation pulse of blue light (about 1800 μmol photons m^{-2} s^{-1}, 600 ms) every 20 s. *rETR* was estimated from the actual PSII photochemical yield measured at different photosynthetic photon flux densities (PPFDs) (Schreiber *et al.,* 2000):

$$rETR=0.42\ (F_m'-F_t)\ F_m'^{-1}\ PPFD$$

where: F_m' and F_t denote the maximum and steady state fluorescence in light, respectively.

The maximal rate of *rETR* ($rETR_{max}$) and photosynthetic efficiency (α) were determined by fitting the rapid light response curve to an exponential function modified from Platt (1980):

$$rETR=rETR_{max}\ (1-e^{-\alpha I\ rETR_{max}^{-1}})$$

where *I* represents the irradiance.

The saturation values (E_k) are determined from the interception point of α value with the maximum photosynthetic rate, which follows the equation (Platt *et al.,* 1980):

$$E_k=rETR_{max}\ \alpha^{-1}$$

φPSII was calculated by the formula (Genty *et al.,* 1989):

$$\varphi PSII=(F_m'-Fs)F_m'^{-1}$$

Where F_s is linked to PSII reaction center closure.

F_v/F_m was calculated by the following formula (Ting and Owens, 1992):

$$F_v/F_m=(F_m-F_o)F_m^{-1}$$

Where F_o is the fluorescence of dark-adapted cells stimulated by a weak probe light immediately following 15 min of darkness; F_m is the maximum fluorescence signal following the closure of all reaction centers by a 600 ms pulse of saturating irradiance.

Statistical analysis

All experiments were performed on three replicates and the data were reported as the average ± standard deviation. One-way analysis of variance (ANOVA) was carried out to test the differences in data ($P<0.05$) between the varied treatments using the Origin 8.0 (OriginLab). Tukey's *post-hoc* tests were used to locate the differences in the significant results.

RESULTS

Cell abundance

Microcystis cell abundance was stable for six days in all treatments, and the exponential growth phase started from then on. The growth curves of the nitrate cultures were similar at the N/P ratios of 100:1, 50:1, 30:1 and 16:1, with no significant differences found in the cell density (one-way ANOVA). The maximum cell abundance was about 4.1×10^6 mL^{-1} obtained at day 13 while it decreased markedly at the N/P ratio of 4:1 from day 12 (Fig. 1a). As for the treatments with NH_4Cl medium, the N/P ratio of 16:1 and 4:1 were superior for the algal growth, which increased rapidly from day 6, and the latter ones occupied the highest cell abundance ($P<0.05$), but nearly fell to 0 at the end of the culture period. The peak number of cell density reached about 3.7×10^6 mL^{-1} at the N/P ratio of 16:1 and 4:1 at day 12 (no significant difference, Fig. 1b) while the other three groups turned to decline since day 9. For the cultures with organic N medium (Fig. 1c), it is interesting to note that the more N-concentrated cultures have the lower cell abundance. The cultures at the N/P ratio of 100:1 and 50:1 declined since day 9. The N/P ratio of 4:1 was beneficial for the algal growth for the first 12 days while the decline phase started from then on. The maximum cell density was about 3.8×10^6 mL^{-1}, appeared at day 14 at the N/P ratios of 16:1 ($P<0.05$). Overall, the maximum cell abundance was significantly high at the nitrate treatments ($P<0.05$), while no significant difference was found between the ammonium and urea treatments (one-way ANOVA).

Pigment concentration

The Chlorophyll-*a* (Chl *a*) and phycocyanin (PC) concentrations in the cultures with different medium have been displayed in Fig. 2. For the *M. aeruginosa* cultured with the $NaNO_3$ medium at different N/P ratios, the Chl *a* concentration and PC concentration were in the trend of rising in the first 12 days and decreased slightly then, except the N/P ratio of 4:1, which exhibited the lowest values. The highest Chl *a* concentration was obtained at the N/P ratio of 100:1 (3.1 mg L^{-1}, $P<0.05$), with the corresponding PC content of 7.5 mg L^{-1}. The peak content of PC (8.3 mg L^{-1}) appeared at day 12 at the N/P ratio of 50:1 ($P<0.05$, Fig. 2a).

Maximum Chl *a* and PC pigment was 2.1 and 8.2 mg L^{-1}, respectively, in the ammonium treatment at the N/P ratio of 16:1 (Fig. 2b). The cultures at the N/P ratio of 4:1 experienced a rapid increase and occupied the highest concentration at day 9, while the significant decline happened then, which was in accord with the highest cell density illustrated in Fig. 1b.

For the cultures with an organic nitrogen source (Fig. 2c), the N/P ratio of 100:1 and 50:1 had a remarkable decline after a short rise, which was related to the decrease of the cell abundance. The highest concentration of Chl *a* and PC were 2.1 and 8.1 mg L^{-1}, appeared at N/P ratio of 16:1 at day 12 ($P<0.05$). The changing trends of the concentration at the N/P ratio of 4:1 was consistent with the results of ammonium medium.

Photosynthetic activity

Rapid light curve

The rapid light curves were measured every three days in each treatment (Fig. 3), together with the corresponding $rETR_{max}$, E_k and α values (Tab. 1). For the treatments with $NaNO_3$ medium, the *rETR* values increased with the increase of nitrogen concentration in the first three days (Fig. 3a). The maximum *rETR* value was 29.11 ($P<0.05$) obtained at the N/P ratio of 100:1 at day 9, with the corresponding E_k and α of 178.84 μmol photons m^{-2} s^{-1} and 0.163, respectively. The group at the N/P ratio of 50:1 presented the maximum *rETR*, E_k and α values (18.62, 116.52, 0.160, $P<0.05$) at day 12 compared to the other N/P ratios, which was consistent with the concentration changes of Chl *a* and PC showed in Fig. 2a. By contrast, the correlation between the *rETR* values and nitrogen concentration was negative in the ammonium treatments (Fig. 3b). The cultures with the lowest N content (N/P ratio of 4:1) had the highest *rETR* values (26.29 at day 9, with the maximum E_k of 169.36, $P<0.05$) and the α values were significantly higher than the other treatments at each time point (0.171, 0.170, 0.155, 0.179, $P<0.05$). However, the most NH_4-concentrated cultures (N/P ratios of 100:1 and 50:1) suffered the last two *rETR* values, which were not applicable to the curve fitting model of Platt (1980) since day 6, resulting in no data showing for E_k and α values (Tab. 1). As for the cultures with the organic nitrogen medium (Fig. 3c), the most beneficial N/P ratio for the algal growth changed during the culture period. Specifically, the cultures with N/P ratios of 100:1 reached the maximum *rETR* value at the first two days (18.74, $P<0.05$). After the first days, the treatments cultured at the N/P ratio of 50:1, 30:1 and 16:1 displayed higher *rETR* values (25.85, 28.29 and 18.44) compared to the other groups successively (day 3, day 6 and day 9, respectively, $P<0.05$). The *rETR* values almost fell to 0 in the cultures at an N/P ratio of 4:1 (5.16 at day 12, $P<0.05$), and no E_k value could be calculated from the model, which indicated that the *Microcystis* cells nearly had no tolerance to the light.

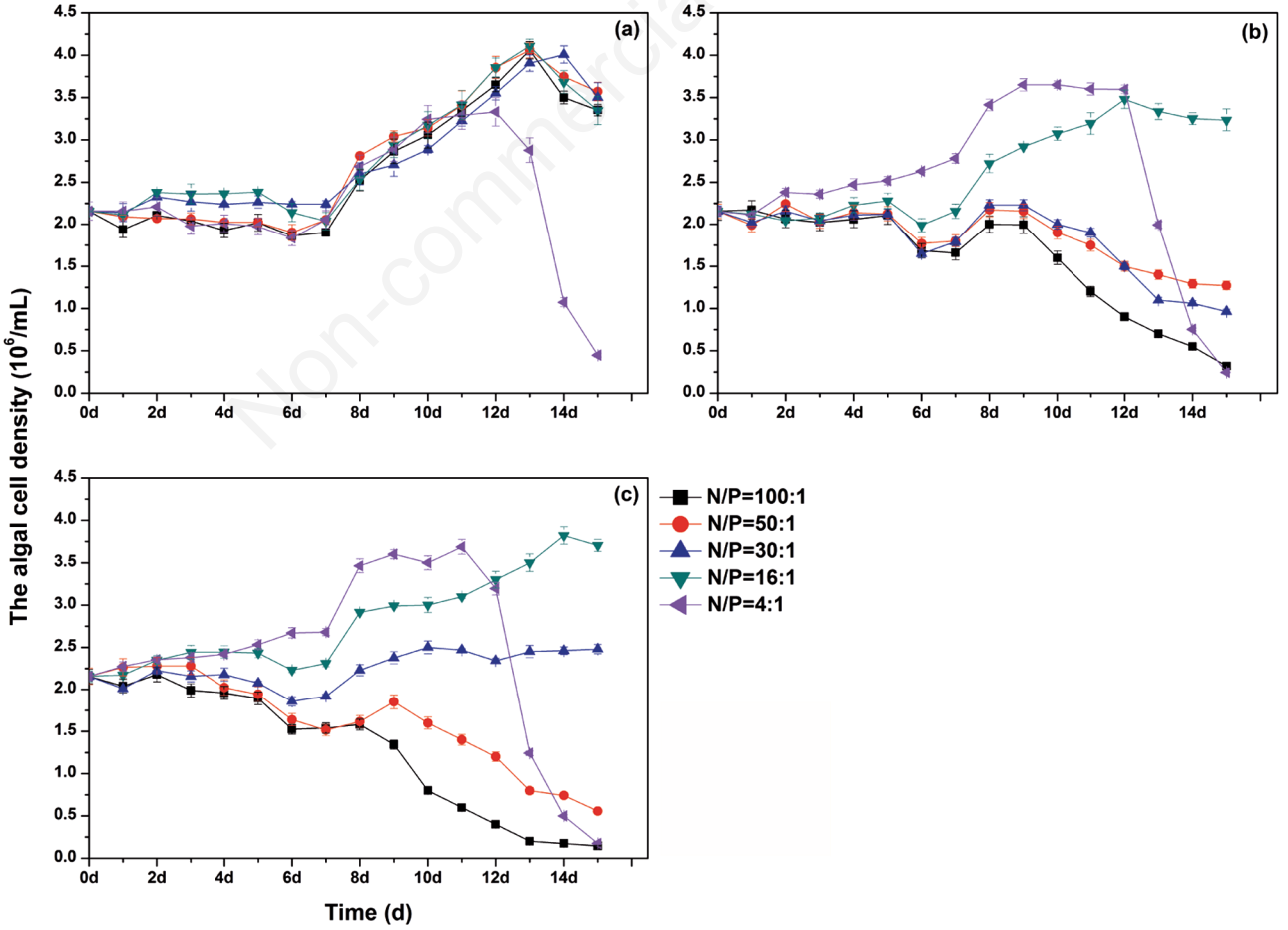

Fig. 1. The cell density of different N/P ratios with $NaNO_3$ (a), NH_4Cl (b) and $[NH_2]_2CO$ (c) medium.

φPSII

The changes of the actual photochemical efficiency of PSII are listed in Tab. 2. The results of each nitrogen source were significantly different among different N/P ratios at each time point ($P<0.05$). Specifically, the treatments with the nitrate medium at the N/P ratios of 100:1, 50:1 and 30:1 possessed the higher values of *φPSII* during the culture period (around 0.3). By contrast, the cultures at the N/P ratio of 16:1 and 4:1 presented low photochemical efficiency gradually, and the lowest *φPSII* value was 0.097 (N/P=16:1, $P<0.05$). By comparison, the values of the ammonium treatments varied a lot among the different N/P ratios. The cultures at the N/P of 4:1 displayed the highest actual photochemical efficiency at each time point ($P<0.05$), and the maximum value was 0.330 (day 10, $P<0.05$). The values of the treatments with N/P ratio of 16:1 and 30:1 had a slight increase from the day 6 while in the more NH_4-concentrated treatments (N/P ratio of 100:1 and 50:1) there was a trend towards decline along with time, and the *φPSII* values at N/P ratio of 100:1 were significantly lower than other groups during the culture period ($P<0.05$). In the treatments with urea the *φPSII* values were stable for the first 8 days in spite of the N/P ratio of 4:1, that decreased and reached the lowest values on day 6 ($P<0.05$). The N/P ratio of 30:1 and 16:1 seized the advantage later on, displaying the highest *φPSII* values ($P<0.05$).

F_v/F_m

The maximum photochemical efficiency of the *M. aeruginosa* was detected after 15 min of dark adaptation (Tab. 3). The F_v/F_m values in the cultures with $NaNO_3$ medium were high (0.4) since the beginning, which indi-

Tab. 1. The values of $rETR_{max}$, E_k and α with different nitrogen sources at each time point, the results were significant different among different N/P ratios (one-way ANOVA, $P<0.05$) Tukey test was used to locate the significant difference.

			100:1	50:1	30:1	16:1	4:1
rETRmax	Day 3	$NaNO_3$	23.34 a	20.86 b	15.27 c	11.65 e	13.22 d
		NH_4Cl	5.43 e	11.14 bc	8.11 d	11.35 b	16.79 a
		$[NH_2]_2CO$	18.74 a	16.78 c	17.62 b	15.59 d	11.39 e
	Day 6	$NaNO_3$	24.32 a	23.33 b	22.62 c	13.81 e	16.84 d
		NH_4Cl	4.23 d	3.55 e	9.42 c	11.57 b	18.99 a
		$[NH_2]_2CO$	14.90 d	25.85 a	21.82 c	22.27 b	5.29 e
	Day 9	$NaNO_3$	29.11 a	21.4 c	23.52 b	10.47 e	13.41 d
		NH_4Cl	4.12 d	4.45 d	10.63 c	13.77 b	26.29 a
		$[NH_2]_2CO$	15.25 c	14.64 d	28.29 a	27.43 b	5.18 e
	Day 12	$NaNO_3$	14.61 c	18.62 a	16.95 b	3.94 e	5.86 d
		NH_4Cl	4.62 e	5.64 d	14.97 c	21.91 a	19.10 b
		$[NH_2]_2CO$	9.51 d	10.18 c	16.22 b	18.44 a	5.16 e
Ek	Day 3	$NaNO_3$	141.41 a	134.43 b	120.25 c	93.51 e	103.92 d
		NH_4Cl	75.43 e	100.23 ab	97.32 d	100.98 a	98.07 c
		$[NH_2]_2CO$	100.99 a	100.19 b	90.52 c	86.13 d	69.94 e
	Day 6	$NaNO_3$	133.87 b	137.34 a	124.67 c	84.78 e	103.24 d
		NH_4Cl	-	-	99.59 c	117.56 a	111.57 b
		$[NH_2]_2CO$	90.99 d	135.33 a	130.52 b	116.91 c	65.17 e
	Day 9	$NaNO_3$	178.84 a	148.72 c	162.10 b	72.86 e	92.28 d
		NH_4Cl	-	-	100.99 c	131.43 b	169.36 a
		$[NH_2]_2CO$	79.60 d	83.05 c	164.53 a	132.71 b	-
	Day 12	$NaNO_3$	107.81 c	116.52 a	113.12 b	63.83 e	73.46 d
		NH_4Cl	-	-	103.80 c	166.78 a	106.92 b
		$[NH_2]_2CO$	69.93 d	82.02 c	157.04 a	140.52 b	-
α	Day 3	$NaNO_3$	0.165 a	0.155 b	0.127 c	0.125 c	0.127 c
		NH_4Cl	0.072 d	0.111 bc	0.083 bcd	0.112 b	0.171 a
		$[NH_2]_2CO$	0.186 b	0.167 d	0.195 a	0.181 c	0.163 e
	Day 6	$NaNO_3$	0.182 a	0.170 c	0.181 ab	0.163 d	0.163 d
		NH_4Cl	-	-	0.095 c	0.098 b	0.170 a
		$[NH_2]_2CO$	0.164 e	0.191 a	0.167 d	0.190 ab	0.081 c
	Day 9	$NaNO_3$	0.163 a	0.144 b	0.145 b	0.144 b	0.145 b
		NH_4Cl	-	-	0.105 b	0.105 b	0.155 a
		$[NH_2]_2CO$	0.192 b	0.176 c	0.172 d	0.207 a	-
	Day 12	$NaNO_3$	0.136 c	0.160 a	0.150 b	0.062 e	0.080 d
		NH_4Cl	-	-	0.144 b	0.131 c	0.179 a
		$[NH_2]_2CO$	0.136 a	0.124 c	0.103 d	0.131 b	-

a-c, indicate observations that are statistically indistinguishable.

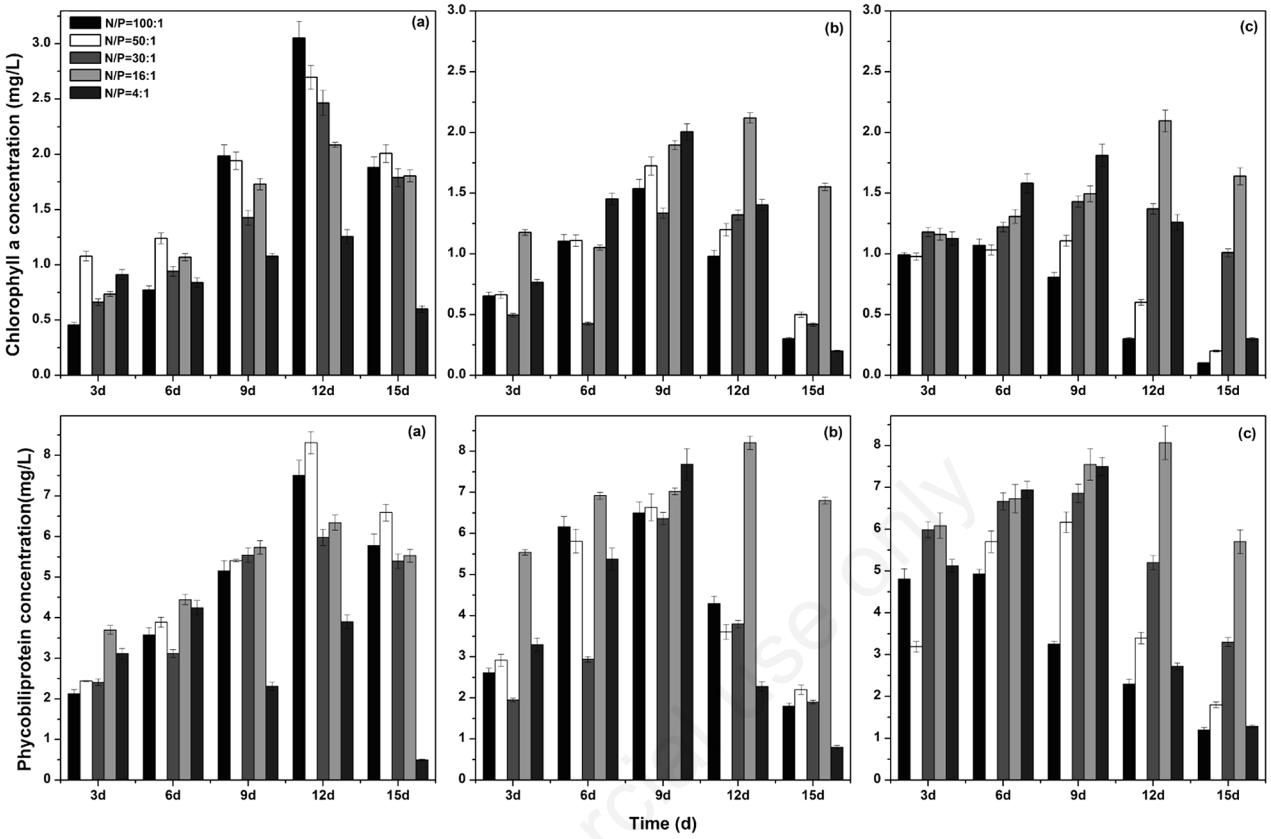

Fig. 2. The chlorophyll-a and phycocyanin concentration of different N/P ratios with $NaNO_3$ (a), NH_4Cl (b) and $[NH_2]_2CO$ (c) medium.

Tab. 2. The actual photochemical efficiency of PSII (φPSII) of the cultures with different nitrogen sources at different N/P ratios. One-way ANOVA was conducted at each N/P ratio at each time point. Tukey's *post-hoc* test was used to locate the significant difference ($P<0.05$).

φPSII		100:1	50:1	30:1	16:1	4:1
$NaNO_3$	2d	0.349±0.017 ab	0.336±0.012 abc	0.359±0.010 a	0.261±0.011 e	0.317±0.016 bcd
	4d	0.329±0.016 a	0.256±0.009 cd	0.298±0.009 ab	0.261±0.010 cd	0.281±0.014 bc
	6d	0.298±0.015 a	0.281±0.010 abc	0.282±0.008 ab	0.233±0.013 d	0.261±0.012 bcd
	8d	0.310±0.016 a	0.287±0.010 ab	0.278±0.008 bc	0.211±0.002 d	0.236±0.012 d
	10d	0.291±0.015 ab	0.309±0.011 a	0.287±0.008 abc	0.181±0.007 e	0.229±0.011 d
	12d	0.281±0.014 b	0.308±0.011 a	0.272±0.008 bc	0.097±0.004 e	0.132±0.007 d
NH_4Cl	2d	0.106±0.0053 e	0.179±0.009 cd	0.185±0.009 bc	0.209±0.010 b	0.315±0.158 a
	4d	0.080±0.004 e	0.148±0.007 bcd	0.158±0.010 bc	0.163±0.008 b	0.280±0.014 a
	6d	0.030±0.002 e	0.116±0.006 cd	0.141±0.001 c	0.171±0.019 b	0.293±0.011 a
	8d	0.020±0.003 e	0.095±0.005 d	0.137±0.007 c	0.196±0.010 b	0.323±0.010 a
	10d	0.017±0.001 e	0.089±0.001 d	0.210±0.011 bc	0.233±0.012 b	0.330±0.013 a
	12d	0.013±0.001 e	0.068±0.003 d	0.172±0.009 bc	0.180±0.009 b	0.260±0.003 a
$[NH_2]_2CO$	2d	0.359±0.018 a	0.328±0.015 abc	0.347±0.011 ab	0.327±0.012 abcd	0.306±0.015 cd
	4d	0.342±0.017 a	0.302±0.014 bc	0.310±0.010 ab	0.265±0.010 d	0.270±0.014 cd
	6d	0.319±0.016 ab	0.341±0.016 a	0.314±0.010 abc	0.279±0.001 cd	0.240±0.012 e
	8d	0.306±0.015 bcd	0.352±0.017 a	0.339±0.011 ab	0.336±0.012 abc	0.201±0.010 e
	10d	0.274±0.014 c	0.270±0.013 cd	0.351±0.012 a	0.311±0.012 b	0.189±0.009 e
	12d	0.236±0.012 c	0.220±0.010 cd	0.338±0.011 a	0.321±0.012 ab	0.080±0.004 e

a-c, indicate observations that are statistically indistinguishable.

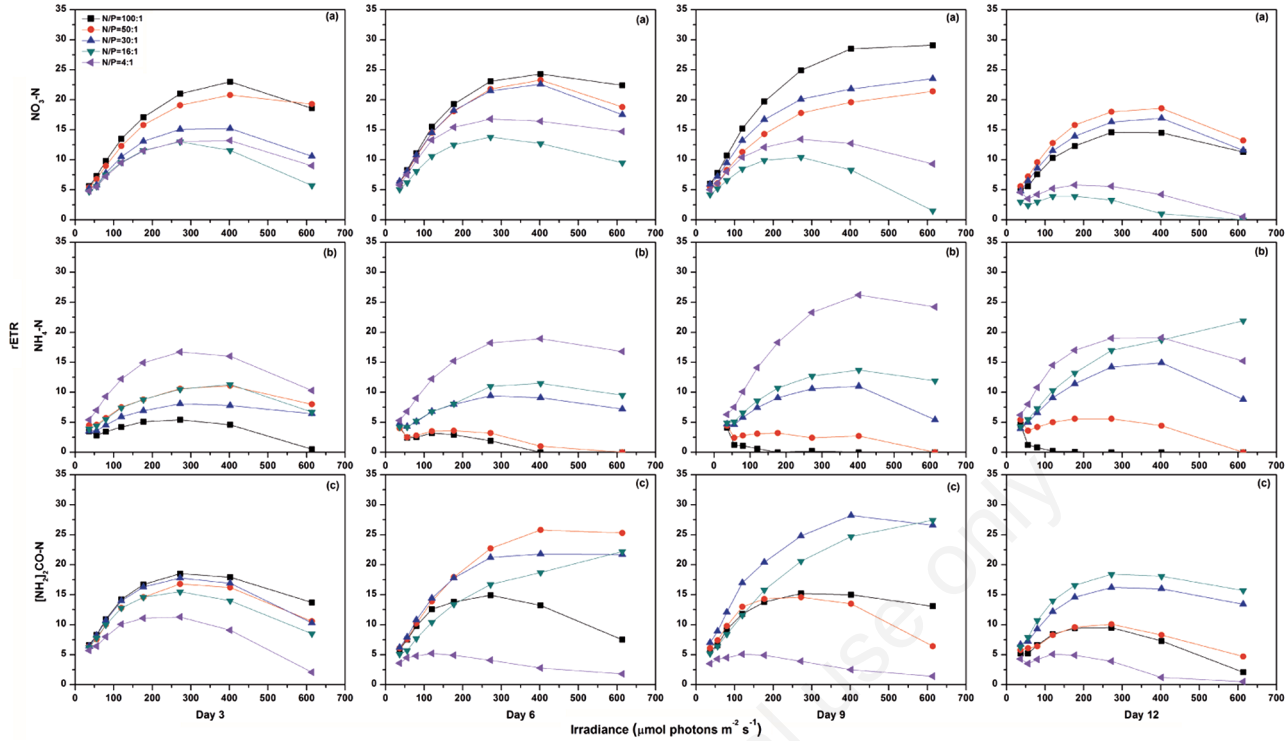

Fig. 3. The rapid light curve (RLC) of different N/P ratios with $NaNO_3$ (a), NH_4Cl (b) and $[NH_2]_2CO$ (c) medium at day 3, day 6, day 9 and day 12.

Tab. 3. The maximun photochemical efficiency of PSII (F_v/F_m) of the cultures with different nitrogen sources at different N/P ratios. One-way ANOVA was conducted at each N/P ratio at each time point. Tukey's range test was used to locate the significant difference ($P<0.05$).

F_v/F_m		100:1	50:1	30:1	16:1	4:1
$NaNO_3$	2d	0.396±0.019 abc	0.409±0.014 ab	0.412±0.012 a	0.334±0.013 e	0.391±0.020 abcd
	4d	0.390±0.020 ab	0.398±0.007 a	0.390±0.011 abc	0.339±0.012 d	0.347±0.017 d
	6d	0.361±0.018 a	0.361±0.013 ab	0.351±0.010 abc	0.317±0.013 abcd	0.333±0.014 cd
	8d	0.365±0.015 a	0.353±0.004 ab	0.343±0.010 abc	0.283±0.011 e	0.325±0.011 bcd
	10d	0.345±0.017 ab	0.357±0.009 a	0.341±0.010 abc	0.267±0.003 d	0.289±0.010 d
	12d	0.338±0.017 ab	0.355±0.012 a	0.322±0.009 abc	0.236±0.009 d	0.250±0.013 d
NH_4Cl	2d	0.354±0.011 bcd	0.377±0.015 b	0.342±0.011 cd	0.368±0.008 bc	0.414±0.007 a
	4d	0.340±0.007 c	0.381±0.012 ab	0.310±0.010 d	0.299±0.007 d	0.386±0.006 a
	6d	0.337±0.012 abc	0.344±0.014 ab	0.258±0.008 d	0.273±0.007 d	0.364±0.009 a
	8d	0.333±0.013 b	0.282±0.010 c	0.249±0.003 e	0.281±0.003 cd	0.377±0.011 a
	10d	0.282±0.004 bc	0.279±0.001 bcd	0.252±0.005 e	0.285±0.006 b	0.346±0.009 a
	12d	0.249±0.012 bcd	0.267±0.011 bc	0.228±0.007 d	0.297±0.007 a	0.268±0.011 b
$[NH_2]_2CO$	2d	0.389±0.019 NSD	0.399±0.020 NSD	0.380±0.019 NSD	0.381±0.019 NSD	0.355±0.018 NSD
	4d	0.376±0.019 abc	0.396±0.020 a	0.389±0.019 ab	0.331±0.018 cd	0.260±0.013 e
	6d	0.342±0.017 abcd	0.370±0.019 a	0.354±0.018 ab	0.372±0.019 abc	0.117±0.016 e
	8d	0.330±0.017 abc	0.330±0.017 abcd	0.352±0.018 ab	0.372±0.019 a	0.117±0.016 e
	10d	0.295±0.015 c	0.288±0.014 cd	0.388±0.019 a	0.356±0.021 ab	0.080±0.004 e
	12d	0.250±0.013 c	0.230±0.012 cd	0.357±0.018 a	0.348±0.017 ab	0.020±0.001 e

a-c, indicate observations that are statistically indistinguishable; NSD, no significant difference.

cated that *M. aeruginosa* was in good conditions. The treatments richer in nitrate (N/P ratios of 100:1, 50:1 and 30:1) had the highest values of F_v/F_m, with no significant difference among treatments. The maximum F_v/F_m value of the treatments with NH_4Cl medium was obtained at the N/P ratio of 4:1 except the day 12 ($P<0.05$). The treatments at the N/P ratio of 100:1 and 50:1 had the higher F_v/F_m values than the other two groups (N/P ratios of 30:1 and 16:1) during all the experiments, opposite to the results of *φPSII*. Nevertheless, there was no significant difference of F_v/F_m among all the N/P ratios with urea medium at day 2. A rapid decline appeared in the cultures at the N/P ratio of 4:1 since day 4, which had the lowest values from then on ($P<0.05$), while the values remained high at the N/P ratio of 30:1 and 16:1 and were significantly higher from day 10 ($P<0.05$).

DISCUSSION

In many parts of the world, aquatic nitrate concentration range from the background level of below 1.0 mg L^{-1} to over 100 mg L^{-1} (Rabalais, 2002; Boyer *et al.,* 2006); while the NH_4^+ concentration may depend upon the urban wastewater treatment process (Leavitt *et al.,* 2006). Moreover, studies have suggested urea may provide 10-50% of the bioavailable N in lakes and river surface waters (Wiegner *et al.,* 2006; Bogard *et al.,* 2012). Preliminary evidences suggest that the effects of nitrogen on algal production and composition may be related to the source and chemical composition of the N added to the lakes (Berman and Chava, 1999; Finlay *et al.,* 2010). In this last studies, significant differences of the *Microcystis* accumulation, pigment contents and photosynthetic responses were found when they were inoculated to different nitrogen medium at varied N/P ratios, which might shed some light on this topic.

In particular, *M. aeruginosa* were adapted to all N/P levels well and kept high cell abundance even at the high nitrate concentration (NO_3/P ratio of 100:1), which was in accordance with the findings from Nalewajko and Murphy (2001). For the cultures with ammonium medium, the optimal NH_4/P ratio for the cell growth was between 4-16, and the sharp decline at the N/P ratio of 4:1 from the day 12 might be explained by the exhaustion of the nutrient after the exponential growth. Although the maximum cell abundance was obtained at the nitrate treatment, the ammonium cultures achieved the peak first (0.11 d^{-1}), which demonstrated that *Microcystis* had the priority to use the ammonium. In the presence of NH_4-N, the growth rate of *Microcystis* should be 4.6 times higher than for NO_3-N (Sirenko, 1972).

The reason may be that the nitrate needs to be converted to ammonium catalyzed by nitrate reductase (NR) and nitrite reductase (NiR) in the cytosol and chloroplast, respectively, before being incorporated into organic compounds (Matthes *et al.,* 1996). However, the toxicity of ammonium is well established in plant and animal systems (Von Wiren and Merrick, 2004). Dai *et al.* (2012) also reported that the *M. aeruginosa* bloom always happened at the low concentration of ammonia in summer, and disappeared with the decrease of ammonia, which may be attributed to the toxic effect of ammonia to *M. aeruginosa* in spring. Therefore, we infer that the reason for the low cell abundance of the NH_4-concentrated cultures (N/P ratios of 100:1, 50:1 and 30:1) in our study may be the ammonium toxicity (Fig. 1b). Furthermore, the energetic advantage of urea may be at least as great as that of NH_4^+ (Berman and Chava, 1999) because urea could be hydrolysed into NH_4^+ and inorganic carbon (C) by urease, encoded by *ureABC* genes (Solomon *et al.,* 2010) or by UALase, a single and fused protein encoded by the *DUR1,2* gene (Haynes and Mokolobate, 2001). In our study, the *Microcystis* growth curves of urea cultures were similar to those of NH_4-N treatments, and N/P ratio favoring algal growth was between 4-30.

Chlorophyll *a* is a critical photosynthetic pigment also for *Microcystis*, and its concentration is related to the growth of the algae itself (Ding *et al.,* 2013), while phycocyanin is one of the major phycobiliprotein in phycobilisomes, which plays an important role in funneling light energy to the underlying PSII reaction centers and would especially decrease under conditions of nutrient depletion (Collier and Grossman, 1994; Prasad and Dubey, 2011). The changes in Chl *a* and PC concentration arose mainly from the variation in *Microcystis* abundance. The treatments at an N/P ratio of 4:1 had the lowest pigment concentration, since the nitrogen concentration is even low (m_N=4 mg/L) compared to the background level of lakes and rivers (m_N=10 mg/L). The optimal N/P ratio for the ammonium and urea treatments to synthesize pigment were both 16:1 in this study, which is in accordance with Redfield's Law (Redfield *et al.,* 1986), and this could be explained by the similar growth curves obtained in our experiment.

Furthermore, the differences of the N forms and concentration resulted in the variable photosynthetic activity. The *φPSII* and F_v/F_m values declined significantly if the microalgae were under stress. The rapid decrease of F_m' or the increasing proportion of closed *PSII* reaction centers would lead to the decline of *φPSII* according to the formula ($\varphi PSII=(F_m'-F_s)/F_m'$) (Wang *et al.,* 2010). It appeared that the *φPSII* values were lower in the treatments where *rETR* values were low, independently from N/P ratio, which provided more evidence for the nutrient limitation theory of algal growth and photosynthesis. Specifically, Chen *et al.* (2009) reported that the accumulation of intracellular nitrate could be the cause of inhibition of *Microcystis* growth and photosynthesis under high nitrate concentration (over 245.1 mg/L). In this study, the con-

centration of NO_3-N was 422.9 mg L^{-1} at N/P of 100:1. As a result, the *rETR*, *φPSII* and F_v/F_m values declined, while at N/P ratio of 50:1 they maintained high values from day 12, suggesting the occurrence of photodamage. Nitrate can regulate NR activity which increases with the increasing of nitrate concentration (Sivasankar and Oaks, 1996), and when nitrite formed by NR is more than the nitrite reduced by NiR, the accumulation of intracellular nitrite will occur, which lead to the inhibition of algal growth and photosynthesis.

As for the ammonium treatments, Zhang *et al.* (2006) indicated that the photosynthetic activity of the microalgae was high when the concentration of the ammonium was between 1.83 and 18.3 mg L^{-1}. Hence, at the N/P ratio of 4:1 in this study, the concentration of NH_4-N was 5.14 mg L^{-1}, with the the maximum values of *rETR*, *φPSII* and F_v/F_m observed, which was conformed to the previous reports. Physiological and genetic research suggested that N assimilation by cyanobacteria is under centralized control in which low cellular quotas of NH_4^+ induce genetic mechanisms (activation of NtcA transcription promoter) that increase the active uptake of dissolved or atmospheric N (Herrero *et al.,* 2004; Flores and Herrero, 2005), thereby we speculated that the low concentration of ammonium is more beneficial for the algal photosynthesis.

Few research has been reported about the effect of organic nitrogen medium on the algal photosynthesis. The maximum photosynthetic electron transport rate changed with the N/P ratios in the urea treatments, the more concentrated treatments had the higher *rETR* values at the beginning. Moreover, the *φPSII* and F_v/F_m values were pretty low at the N/P ratio of 4:1, which might be due to the decrease of the efficiency of electron transport from the primary quinone-type acceptor (QA^-) to QB (Antal *et al.,* 2009; Petrou *et al.,* 2012), or to the decline of the D_1 protein concentration, which is low under nutrient deficient conditions (Steglich *et al.,* 2001). Huang *et al.* (2014) also reported that low urea concentrations did not sustainably promote the growth, photosynthesis and toxin production of the *M. aeruginosa*. The urease is present in live microbial cells, which determines the hydrolysis rate of urea (Fisher *et al.,* 2016). However, during this process, CO_2 is released by intracellular urease (Flores and Herrero, 2005), which might change the pH of the solutions (Haynes and Mokolobate, 2001), having a significant impact on the urease activity, as well as on the CO_2 fixation rate of photosynthesis (Klemer *et al.,* 1982).

CONCLUSIONS

Overall, our observations provide insight into the effects of nitrogen forms (inorganic and organic) and varied N/P ratios on the biomass and photosynthetic activity of *M. aeruginosa* FACHB-905. Significant differences of *Microcystis* numbers, pigment contents and photosynthetic responses revealed that: i) *M. aeruginosa* can adapt to medium-high nitrate concentrations (N/P=30-50), while too-high concentrations (N/P=100:1) would cause an intracellular nitrate inhibition, which induce a decrease of the photosynthetic activity; ii) low concentration of NH_4-N (N/P=4) would favor the *M. aeruginosa* growth and photosynthesis, with the maxmium *rETR,* E_k, *α, φPSII,* F_v/F_m values of 26.29, 169.36, 0.179, 0.330 and 0.414, respectively, while too high NH_4/P ratio (>16) would rise the ammonium toxicity of algal cells and affect the N assimilation; iii) the organic nitrogen (urea) is a bioavailable form of N and in our experiments the highest growth of *M. aeruginosa* was observed at N/P ratio between 16-30; while the values of the photosynthetic parameters (*rETR, φPSII* and F_v/F_m) were pretty low (almost 0) at the N/P ratio of 4:1. Thus, we speculate that the nitrogen chemical forms and the N/P ratios are two important factors for cyanobacterial growth and photosynthetic activity. The control of nitrogen inputs during the algal blooms should not be ignored. However, further studies like the antioxidant system activity assay and the molecular response should be carried out to clarify the mechanisms of the photosynthetic response to nutrient stress.

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