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Physiological effects of nitrate, ammonium, and urea on the growth and microcystins contamination of *Microcystis aeruginosa*: Implication for nitrogen mitigation

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ABSTRACT

The effects of three commonly bioavailable nitrogen (N) sources (nitrate, ammonium, and urea) on regulating the growth and microcystins (MCs) production of *Microcystis aeruginosa* (*M. aeruginosa*) at environmentally relevant concentrations were investigated from a physiological perspective. Changes in amino acid quotas as well as the transcripts of target genes associated with N metabolism (*ntcA*, *pipX* and *glnB*) and toxin formation (*mcyA* and *mcyD*) were determined. Results indicated that increases in nitrate and urea concentrations enhanced *M. aeruginosa* growth, but high ammonium concentration (7 mg-N/L) suppressed the growth. The total intracellular MCs (IMCs) content was well correlated (0.65, $p < 0.001$) to amino acids (the sum of methionine, leucine, serine, alanine, arginine, glutamic acid, and aspartic acid) associated with MCs production. Ammonium favors amino acid synthesis in *M. aeruginosa*, thus cells grown under high concentrations of ammonium (7 mg-N/L) had sufficient precursors for MCs production, which might lead to higher IMCs. Both high and low ammonium concentration resulted in high total extracellular MCs (EMCs) level in water, despite of their different mechanisms. These results indicated that mitigation of nitrogen in eutrophic waters should be very cautious of unexpected risks, as the reduction of ammonium may have the risk of stimulating *M. aeruginosa* growth or increasing EMCs levels.

1. Introduction

Freshwater bodies are often receiving excessive nitrogen (N) and phosphorus (P) loads derived from intensive anthropogenic activities, including domestic sewage, agricultural fertilizer, industrial discharge, and fossil fuel combustion, which facilitate the occurrence of harmful cyanobacterial blooms globally (Huisman et al., 2018; Yi et al., 2017). Traditionally, P is considered the primary limiting nutrient in the proliferation and expansion of cyanobacterial blooms (Schindler, 1974) based on the premise that N supplies can be met by N_2 fixation (Harke et al., 2016a). For decades, P reduction has been considered the most effective strategy for bloom management (Conley et al., 2009); however, increasing evidences have highlighted the importance of N in freshwater eutrophication (Smith and Schindler, 2009; Yu et al., 2019). Given the expanding blooms of non-N_2 fixer *Microcystis* in many eutrophic systems (Bridgeman et al., 2013; Guan et al., 2018), the role of external N in the global proliferation of cyanobacterial blooms seems certainly underestimated.

Several cyanobacterial species can produce neurotoxic and hepatotoxic compounds (Jeong et al., 2017; Penn et al., 2014). Microcystins (MCs), which are mainly produced by the *Microcystis*, *Anabaena*, *Nostoc*, *Oscillatoria*, and *Planktothrix* genera (Nishizawa et al., 2000), can have detrimental effects on aquatic organisms, wildlife, livestock, and even induce human fatalities in case of chronic exposure (Azevedo et al., 2002; Song et al., 2007). The MCs family currently includes at least 246 hepatotoxins containing cyclic heptapeptides (Meriluoto et al., 2017), whose synthesis is directed by the microcystin synthetase (*mcy*) gene

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cluster composed of two putative operons (*mcyABC* and *mcyDEFGHIJ*) (Tillett et al., 2000). The generalized MCs structure consists of five constant amino acids (alanine, leucine, arginine, glutamic acid, and aspartic acid) and two variable L-amino acids. Among the variants, microcystin-LR (MC-LR) is the most common and toxic (Carmichael, 1992), with leucine (L) and arginine (R) the two variable L-amino acids.

As an essential macronutrient, N not only plays an important role in cyanobacterial growth but also in a series of physiological processes, including MCs production (Horst et al., 2014). Nitrate and ammonium are the most commonly available N sources in freshwater, while urea content has been increasing due to its frequent application in agricultural fertilizer and aquaculture (Ahnen et al., 2015; Glibert et al., 2006). *Microcystis* exhibits different growth statuses and MCs levels following exposure to different forms and concentrations of N (Li et al., 2016; Wu et al., 2015). Ginn et al. (2010) analyzed the regulation of MC synthesis by N in *Microcystis aeruginosa* (*M. aeruginosa*) PCC 7806, and discovered that NtcA, the global N regulator encoded by the *ntcA* gene, could bind to the bidirectional *mcyA*/*D* promoter region and function as an activator of *mcy* gene transcription. In cyanobacteria, N control is tightly mediated by NtcA with the assistance of N regulator protein P_{II} (*glnB* gene product) and its interaction protein PipX (*pipX* gene product) (Frias et al., 1993; Herrero et al., 2001). Ambient N is transported into cells and converted into ammonium through different pathways, and then enter into the glutamine synthetase (GS) and glutamate synthase (GOGAT) pathway incorporating with 2-oxoglutarate (2-OG) for eventual assimilation (Muro-Pastor and Florencio, 2003). Once the N is assimilated, aminotransferases can transfer the amino group from glutamate to other carbon backbones to form additional amino acids. When cells are in the condition of low N levels, PipX dissociates from the phosphorylated P_{II} protein and interacts with NtcA-2-OG, affecting the expressing of N assimilation related gene. When cells are in the condition of high N levels, PipX is bound with the unphosphorylated P_{II} instead of NtcA, leading to the inhibition of N uptake (Esteves-Ferreira et al., 2018; Llácer et al., 2010). Thus, changes in ambient N will affect the transcription of *ntcA* and other N metabolism-associated genes, and possibly the *mcy* gene cluster.

Amino acids are vital precursor substances participating in MCs biosynthesis (Tillett et al., 2000). Previous studies have primarily concentrated on changes in *Microcystis* growth and MCs production in response to externally added amino acids (Dai et al., 2009; Yan et al., 2004). *Microcystis* can uptake and utilize several amino acids for MCs synthesis, though the addition of leucine and arginine both inhibit MCs production (Yan et al., 2004). However, little is known about changes in the composition and content of intracellular amino acids or, the relationship between intracellular amino acid and MCs production when *Microcystis* is under different N conditions.

In this study, an axenic *M. aeruginosa* strain was selected as a model organism. Changes in *M. aeruginosa* growth status, photosynthetic activity, N utilization, MCs production and amino acid quotas were monitored, along with the transcription of target genes associated with N metabolism and toxin formation, under different bioavailable nitrogenous compound supplies. The main objective of this research was to discover the physiological effects of different N sources on regulating the growth and MCs production of *M. aeruginosa* at environmentally relevant concentrations, from both phenotypic and molecular aspects. The results could be of value to provide a scientific basis for N management, including the N species and levels, in eutrophic waters.

2. Materials and methods

2.1. Organism and its cultivation

M. aeruginosa FACHB-905 (hereafter *M. aeruginosa*) was purchased from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). The unicellular strain is demonstrated to mainly produce MC-LR (Dai et al., 2008; Lei et al., 2015). It was pre-cultured in BG-11 medium (Table S1 in Supporting Information) with an adjusted pH of 8.2 for at least 30 d to maintain a good growth status under constant conditions (25 °C, illumination of 35 μmol/(m^2·s) on 12: 12 h light/dark cycle). Cultures were gently agitated by hand three times daily.

The sources of N used here were nitrate (sodium nitrate), ammonium (ammonium chloride), and urea. Pre-cultures in the exponential phase were harvested by centrifugation (5000 rpm, 15 min, 4 °C), and rinsed twice with sodium bicarbonate solution (15 mg/L). The cells were re-suspended in N-free BG-11 medium for 3 d to exhaust intracellular N, which was introduced by cells from the pre-cultured N-rich BG-11 medium. This procedure has been commonly used in previous studies, and no adverse effects were observed in the subsequent experiments (Dai et al., 2009; Liu et al., 2019). During algal blooms in nature water bodies, N is largely consumed and its concentration often decreases sharply. Thus, we adopted batch cultures in this study. They were performed in 2000 mL conical flasks, containing 1000 mL modified BG-11 medium with an initial cell density of 5×10^5 cells/mL. Three levels of each N species (0.5, 3 and 7 mg-N/L) were chosen and the pH was set to 8.2 ± 0.2 according to our long-term field investigations (1998–2016) in Taihu Lake, China, where severe cyanobacterial blooms occur frequently. The pH was maintained by adding N-[tris(hydroxymethyl)methyl-3-amino] propanesulfonic acid. Cultures were gently agitated by hand three times daily, and rearranged randomly to reduce disturbances caused by uneven light intensity in the incubator. The 3 mg-N/L of every source of N was used as the control (standard). All experiments were conducted in triplicate.

2.2. Analysis methods

2.2.1. Cell density, Fv/Fm, and N concentration

Cell density, maximum quantum efficiency of photosystem II (*Fv*/*Fm*), and N concentrations in culture solutions were analyzed every two days. Cell density was counted using a hemocytometer under a microscope (OLYMPUS CX41RF, Japan). The specific growth rate (μ) was calculated as $\mu\ (d^{-1}) = \ln(N_2)\text{-}\ln(N_1)/(t_2\text{-}t_1)$, where N_2 and N_1 represent the cell numbers at time t_2 and t_1, respectively. Response of photosynthetic yield to different N sources was analyzed by measuring the *Fv*/*Fm* using a PHYTO-PAM fluorometer (WALZ, Germany), which has been commonly used as a sensitive diagnostic of N-limitation for *Microcystis* (Simis et al., 2012; Harke and Gobler, 2013). Before measurement, cultures were kept in darkness for 10 min to allow all reaction centers to open. *Fv*/*Fm* was calculated as $Fv/Fm = (Fm\text{-}Fo)/Fm$, where *Fm* and *Fo* are the maximum and minimum fluorescence yields of the dark-adapted cells, respectively.

50 mL of culture sample was filtered through GF/C glass fiber filters (pore size 1.2 μm, Whatman, UK). The filtrates were used for determination of N concentration in the culture solutions. Nitrate and ammonium concentrations were determined by the phenol disulfonic acid method and Nessler method, respectively (Huang, 2000). Samples used for urea determination were incubated with urease in a 50 °C water bath to convert urea N into ammonium quantitatively (McCarthy, 1970), and then determined by Nessler

method. The uptake rate (V) and utilization efficiency (η) of N were calculated by V (pg/cell·d) = $(C_t\text{-}C_0) \times 10^6/(t \times n)$ (Downing et al., 2005), and $\eta = (C_t\text{-}C_0)/C_0 \times 100\%$, respectively, where C_0 and C_t are the initial and final concentrations (mg/L) of every N substrate in the medium, t is the interval time (d), and n is the cell numbers at time t.

2.2.2. MCs concentration

Extracellular MCs (EMCs) and intracellular MCs (IMCs) concentrations were analyzed using an enzyme-linked immunosorbent assay (ELISA) kit (Beacon, USA), at the mid-exponential and mid-stationary phases, respectively. EMCs represented the MCs in the medium, while IMCs were the toxins in living cells. Depending on cell density, the culture samples (5–10 mL) were centrifuged at 10,000 rpm for 10 min, at 4 °C. The supernatants and sediments were used to estimate EMCs and IMCs concentrations. The EMCs were analyzed according to the manufacturer's instructions using a BioTek Epoch Microplate Reader (USA). To extract IMCs, 1 mL of double-distilled water was added to the sediments to re-suspend the cells. The cell suspension was firstly lysed by the freeze-thaw cycle, and then centrifuged at 10,000 rpm for 10 min, at 4 °C. The supernatants were processed following the same method as used for the EMCs. The coefficient of the calibration curve was higher than 0.99, and the lower detection limit was set at 0.1 μg/L. The recovery of the spiked samples was 94.3 ± 2.1% ($n = 5$).

2.2.3. Protein and amino acid concentrations

Protein and amino acid concentrations were determined at mid-exponential and mid-stationary phases, respectively. 50 mL of culture sample was harvested by centrifugation (8000 rpm, 10 min, 4 °C), with cells then processed using an algae protein extraction kit according to the manufacturer's instructions (BestBio, China). The concentration of extracted protein was determined using the bicinchoninic acid method (Pierce® BCA Protein Assay Kit, Thermo Scientific, USA). The coefficient of the calibration curve was higher than 0.99, with a detection range of 20–2000 μg/mL.

Another 50 mL of culture sample was harvested by centrifugation (8000 rpm, 10 min, 4 °C), with the supernatant then discarded. The cells were re-suspended with 16 mL of 6 mol/L hydrochloric acid and transferred to a hydrolysis tube. The tubes were degassed using a vacuum for 30 min followed by N_2-sparging, and then hydrolyzed at 110 °C for 24 h. Hydrolysates were adjusted to 50 mL with distilled water using a volumetric flask. Afterwards, 1 mL of solution was taken and processed by deacidification and dehydration in vacuum. This procedure was repeated three times, and the residues were re-dissolved in 1 mL of 0.02 mol/L hydrochloric acid. After that, 0.5 mL of the re-dissolved solution was taken, and thoroughly mixed with 0.25 mL of 1 mol/L triethylamine acetonitrile and 0.25 mL of 0.1 mol/L phenylisothiocyanate acetonitrile. The mixture was rested for 1 h at room temperature, then violently shaken after adding 2 mL of n-hexane, and again rested for 10 min. Finally, the lower suspension was filtered, and the filtrate was used for determination of amino acid concentration by using a high-pressure liquid chromatograph (HPLC).

A standard mixture of sixteen different amino acids (Sigma, USA) was sampled using a HPLC (Agilent 1260, USA) equipped with an ODS column (5 μm, 4.6 × 250 mm, Agilent, USA) and an ultraviolet detector at 254 nm. The mobile phase included phase A (0.1 mol/L sodium acetate containing 3% acetonitrile, pH 6.5) and phase B (80% acetonitrile). The flow rate of the mobile phase was 1 mL/min, with a gradient profile of 0 min and 100% A, 14 min and 85% A, 29 min and 66% A, 30 min and 100% B, 37 min and 100% B, 37.1 min 100% A, and 45 min and 100% A. The injection amount was 10 μL, and the column temperature was 40 °C. The amino acids in the samples were identified and quantified by comparing the retention times and absorption peak areas to the standards. The HPLC profile of the standard mixture was presented in Fig. S1 in Supporting Information. The limits of quantification (LOQ) for every amino acid, and the parameters of the calibration curves used for determination of amino acids concentrations were shown in Table S2 in Supporting Information, with the coefficients of all calibration curves higher than 0.99.

2.2.4. RNA extraction, reverse transcription, and qPCR

Cells from a 50 mL sample at mid-exponential and mid-stationary phases, respectively, were separated by centrifugation (8000 rpm, 10 min, 4 °C), and used for extraction of total RNA using a RNeasy Plant Mini Kit (QIAGEN, Germany) in accordance with the manufacturer's instructions. The concentration and purity of total RNA were determined spectrophotometrically with a NanoDrop One (Thermo Scientific, USA), and its integrity was checked on a 1.5% agarose gel. Elimination of residual genomic DNA from the RNA samples and reverse transcription were performed using a PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan) following the manufacturer's instructions.

Copies of target genes, *ntcA*, *pipX*, *glnB*, *mcyA*, and *mcyD*, were quantified by quantitative polymerase chain reaction (qPCR), with 16S rRNA selected as the housekeeping gene. The qPCR assays were performed in triplicate on a BioRad CFX 96 PCR system. All reactions contained 12.5 μL of 2 × SYBR *Primer EX Taq* II (Takara, Japan), 0.5 μL of each forward and reverse primer (Table 1), and 2 μL of template cDNA to a final volume of 25 μL with sterile water.

The qPCR program consisted of an initial pre-heating step for 30 s at 95 °C, and 40 cycles of 5 s at 95 °C, 10 s at 55 °C, and 20 s at 72 °C. Melting curve analyses were performed by gradually increasing the temperature from 70 °C to 95 °C with a 0.5 °C/s increment. The relative expression ratios were calculated using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001) and the control (3 mg-N/L) as reference to determine changes in gene expression under different N concentrations.

2.3. Statistical analysis

All statistical analyses were conducted using SPSS 22.0 for Windows (SPSS Inc., Chicago, IL, USA). Data were presented as means ± standard deviation (SD). Differences in cell density, *Fv*/*Fm*, specific growth rate, N uptake rate, MCs concentration, protein quotas, and relative expression ratios of target genes among different N treatments were tested by one-way analysis of variance (ANOVA). The level of significance was set at $p < 0.05$. The relationships among specific growth rate, N uptake rate, MCs concentration and protein quotas were analyzed using Pearson's correlation.

3. Results

3.1. Growth, photosynthetic activity, and N utilization of M. aeruginosa

M. aeruginosa growth was better stimulated under the nitrate treatments than the ammonium and urea treatments (Fig. 1). Cells grown under 0.5 mg-N/L of each different N form reached the stationary phase within the initial 6 d, after which cell densities were significantly lower than the control (3 mg-N/L) (ANOVA, $p < 0.05$). Cell density at the 3 mg-N/L of ammonium treatment was higher than those at the other ammonium treatments during 12-d incubation period (Fig. 1B), presenting a significantly higher specific growth rate (μ) in both the exponential and stationary phases (Table S3 in Supporting Information; ANOVA, $p < 0.05$). Compared to the nitrate and urea treatments, cell density of *M.*

Table 1
List of primers used in this study.

Target gene	Primer sequence (5′ - 3′)	Reference
16S rRNA	Forward: GCCGCRAGGTGAAAMCTAA Reverse: AATCCAAARACCTTCCTCCC	Neilan et al. (1997)
ntcA	Forward: TGCAGGGTTTGTCCTCGCGG Reverse: CCCGGATGCCATCGGTGGTG	Pimentel and Giani (2014)
glnB	Forward: ACCGTCTCCGAAGTTAGAGGTTT Reverse: AGATTTTACCGTCTCCGATTTCAC	This study
pipX	Forward: GAAACCTATCTCAATCATCCCACTT Reverse: TTCCACCAAAAGACGAGCA	This study
mcyA	Forward: AAAATTAAAAGCCGTATCAAA Reverse: AAAAGTGTTTTATTAGCGGCTCAT	Hisbergues et al. (2003)
mcyD	Forward: GGTTCGCCTGGTCAAAGTAA Reverse: CCTCGCTAAAGAAGGGTTGA	Kaebernick et al. (2000)

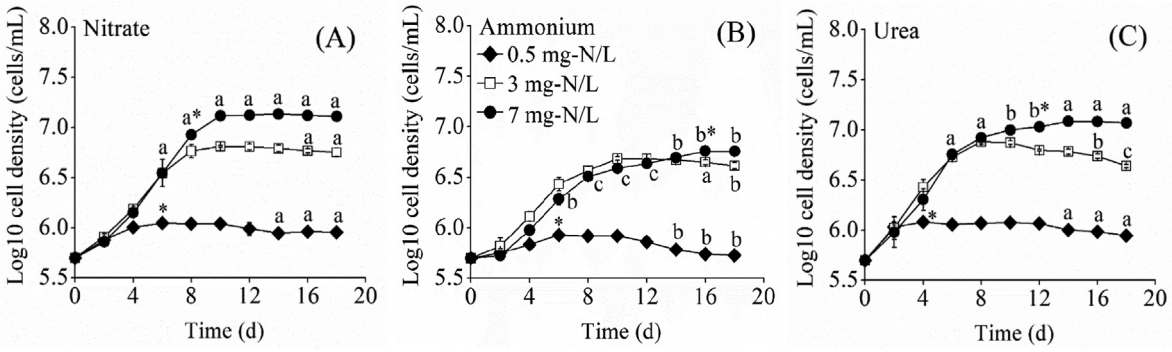

Fig. 1. Growth curves of *M. aeruginosa* under different N treatments (form and concentration). Different letters indicate statistically distinguishable observations under different N sources of the same concentration (ANOVA, $p < 0.05$). Asterisk indicates significant differences compared to the control (ANOVA, $p < 0.05$), and the following days present the same results although they are not marked.

aeruginosa cultured in the 7 mg-N/L ammonium was significantly lower since day 6 (ANOVA, $p < 0.05$). After 14 d of growth, the maximum cell densities in the nitrate and urea treatments occurred at 7 mg-N/L, and were 1.37×10^7 and 1.22×10^7 cells/mL, respectively. The maximum cell density in ammonium treatment was also found at 7 mg-N/L but on day 16, reaching a peak of 5.75×10^6 cells/mL, which was almost half of that observed in nitrate treatment.

The photosynthetic activity of *M. aeruginosa* under different N treatments was presented in Fig. 2. At the 0.5 mg-N/L, *Fv*/*Fm* was significantly lower for all N forms than the control at the initial cultivation (ANOVA, $p < 0.05$), and kept decreasing to be lower than 0.2 on day 12. In the 3 and 7 mg-N/L treatments, *Fv*/*Fm* increased at first and then decreased with time for all N forms. At the end of cultivation, *Fv*/*Fm* was statistically higher under 3 and 7 mg-N/L urea treatments, compared to the nitrate and ammonium treatments with the same N concentrations (ANOVA, $p < 0.05$).

As shown in Fig. 3, N was quickly consumed by *M. aeruginosa* in all 0.5 mg-N/L treatments, with most N exhaustion after 12 d of incubation. In the 3 and 7 mg-N/L treatments, the concentration of nitrate declined slowly during the first 4 d of incubation and decreased sharply afterwards. The final N utilization efficiency (η) in almost all treatments was nearly 100%, except in the 7 mg-N/L ammonium treatment where it was 94.8%. For all treatments, the N uptake rate (V) significantly decreased from the exponential phase to stationary phase (Table S3 in Supporting Information; ANOVA, $p < 0.05$). Additionally, the ammonium treatments presented higher V than the nitrate and urea treatments, in both the exponential and stationary phases.

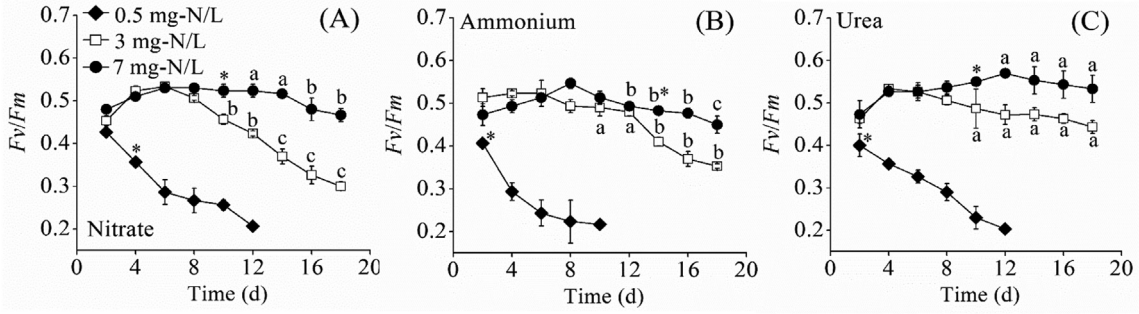

Fig. 2. Photosynthetic activity of *M. aeruginosa* under different N treatments (form and concentration). Different letters indicate statistically distinguishable observations under different N sources of the same concentration (ANOVA, $p < 0.05$). Asterisk indicates significant difference compared to the control (ANOVA, $p < 0.05$), and the following days present the same results although they are not marked.

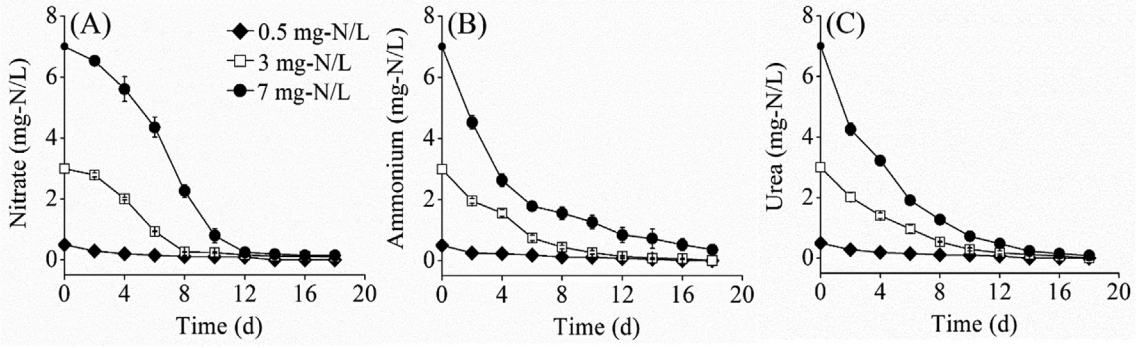

Fig. 3. Changes in N concentrations along time in culture media with different N initial contents. (A) nitritae treatment; (B) ammonioum treatment; and (C) urea treatment.

3.2. Differential MCs concentration among treatments

As shown in Fig. 4A, in nitrate treatments, the IMCs were significantly higher under 0.5 and 3 mg-N/L treatments than 7 mg-N/L treatment in the exponential phase (ANOVA, $p < 0.05$), while they exhibited no significant differences in the stationary phase. IMCs were promoted by the increase in ammonium concentration from 0.5 to 7 mg-N/L, peaking at 45.16 and 54.52 fg/cell in the exponential and stationary phases, respectively. Additionally, there were no significant differences in IMCs concentration in the urea treatments (ANOVA, $p > 0.05$). Following algal growth from the exponential to stationary phase, IMCs decreased significantly in the 0.5 and 3 mg-N/L nitrate treatments, but increased significantly in the 0.5 mg-N/L ammonium treatment (ANOVA, $p < 0.05$).

The EMCs concentrations were significantly higher under 0.5 and 7 mg-N/L than 3 mg-N/L nitrate treatment in the exponential phase (ANOVA, $p < 0.05$), but exhibited no significant differences in the stationary phase (Fig. 4B). In contrast, cells cultured in 0.5 and 7 mg-N/L ammonium showed significantly higher EMCs levels than that of 3 mg-N/L ammonium treatment (ANOVA, $p < 0.05$). In urea treatments, the EMCs concentrations were significantly higher under 0.5 mg-N/L than 3 and 7 mg-N/L treatments in both exponential and stationary phases (ANOVA, $p < 0.05$). Additionally, both IMCs and EMCs were negatively correlated with specific growth rate, and were positively correlated with N uptake rate (Fig. S2 in Supporting Information; $p < 0.05$, $n = 54$).

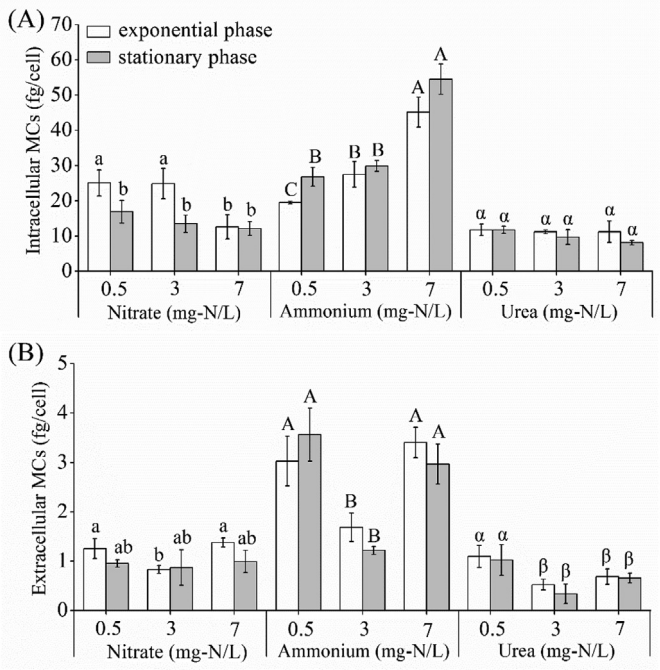

Fig. 4. Intracellular and extracellular MCs concentrations under different N treatments (form and concentration). Different letters indicate statistically distinguishable observations (ANOVA, $p < 0.05$).

3.3. Differential amino acid and protein quotas among treatments

Among the amino acids, methionine, leucine, lysine, phenylalanine, and tyrosine were more abundant than the others in most treatments, especially ammonium (Fig. 5A). The cells cultured in ammonium had higher contents of amino acids associated with MCs synthesis (methionine, leucine, serine, alanine, arginine, and glutamic acid) than the cells cultured in nitrate and urea. Aspartic acid, which also participates in MCs synthesis, was more abundant under nitrate than the other N treatments. In both exponential and stationary phases, the quota of each amino acid showed a decreasing trend with the increase in N concentration in the nitrate and urea treatments; however, for ammonium treatment, all amino acid quotas increased at first and then decreased except leucine. Following algal growth from the exponential to stationary phase, all amino acid quotas declined in most treatments (Fig. 5A). Furthermore, IMCs concentrations were positively correlated with the content of amino acids associated with MCs synthesis (Fig. 5B; $R^2 = 0.645$, $p < 0.001$, $n = 17$).

The variations in protein quota were similar to those in amino acid quota in all treatments (Fig. 5C). Moreover, the protein quota was significantly higher in the exponential phase than in the stationary phase (ANOVA, $p < 0.05$) in all treatments, except for the 0.5 mg-N/L nitrate treatment. Protein quotas were negatively associated with specific growth rate, and were positively correlated with N uptake rate (Fig. S2 in Supporting Information; $p < 0.05$, $n = 54$).

3.4. Differential transcripts of target genes among treatments

The effects of nitrate on the expression of N metabolism-associated genes (*ntcA*, *pipX*, and *glnB*) and MCs biosynthesis-associated genes (*mcyA* and *mcyD*) were similar (Fig. 6A). The transcripts of all target genes were significantly higher under 0.5 mg-N/L nitrate treatment than under 7 mg-N/L treatment (ANOVA, $p < 0.05$), in both the exponential and stationary phases. Compared with the control (3.0 mg-N/L), the transcription of *pipX*, *glnB*, and *mcyD* in the exponential phase, and *glnB* in the stationary phase were significantly up-regulated under 0.5 mg-N/L nitrate treatment (ANOVA, $p < 0.05$).

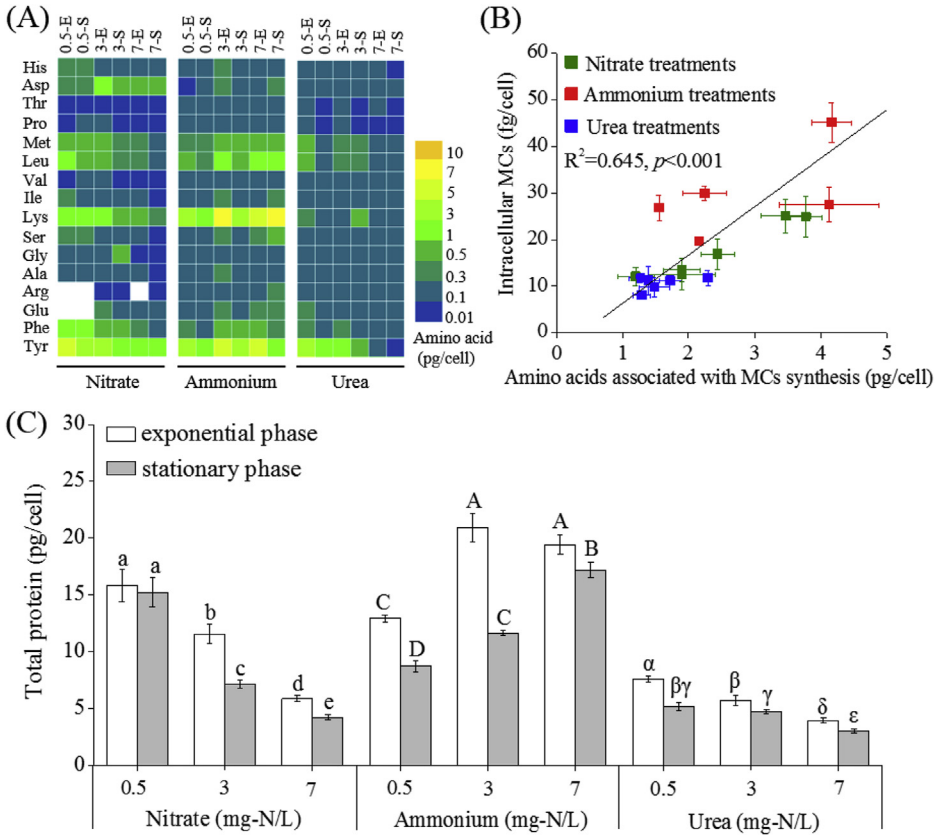

Fig. 5. Amino acids (A) and protein (C) quotas under different N treatments (form and concentration), and the relationship between intracellular MCs and amino acids associated with MCs synthesis (B). E and S represent the exponential and stationary phases, respectively. The white boxes in the heatmap indicate that the amino acid was below the detection limit. The content of amino acids associated with MCs synthesis is the sum of methionine, leucine, serine, alanine, arginine, glutamic acid, and aspartic acid. Different letters indicate statistically distinguishable observations (ANOVA, $p < 0.05$).

The expressions of *ntcA*, *pipX*, and *glnB* were down-regulated with the increase in ammonium and urea concentrations (Fig. 6B and C). Compared to 0.5 mg-N/L, the transcription of *ntcA* was notably down-regulated under 7 mg-N/L ammonium and urea treatments, in both exponential and stationary phases (ANOVA, $p < 0.05$). Higher transcripts of *mcyA* and *mcyD* were detected in the 0.5 mg-N/L ammonium treatment; however, the transcripts of *mcyA* and *mcyD* showed no significant differences under the different urea concentrations.

4. Discussion

4.1. Effects of different nitrogen forms on M. aeruginosa growth

For cyanobacteria, exogenous N entering the cells must be converted to ammonium for assimilation (Esteves-Ferreira et al., 2018), thereby making ammonium the most energetically favorable source of N (Britto et al., 2001). Urea also has energetic advantages as a source of N because each urea molecule can be hydrolyzed into two ammonium molecules (Donald et al., 2011). Therefore, *M. aeruginosa* should grow better in ammonium and urea than in nitrate. However, our results showed that *M. aeruginosa* growth was not closely related to the form of N but to its concentrations. Cells cultured in the different N sources at 0.5 mg-N/L experienced N deprivation due to the exhaustion of N in batch culture, as indicated by the sharply declined cell density, persistently decreased *Fv*/*Fm* values and presence of chlorosis (yellow and turbid cultures). Nevertheless, previous studies conducted in continuous culture report that *Microcystis* could grow well under N-limiting conditions (Downing et al., 2005). These findings suggested that, when algal blooms occurred without sufficient exogenous nitrogen input, an environmentally relevant N concentration of 0.5 mg-N/L could not sustain long term growth of *M. aeruginosa*, no matter which nitrogenous compound was used as the N source.

The increase in nitrate concentration exerted significant promotion effects on *M. aeruginosa* growth (Fig. 1). In the nitrate treatments, the cells grew slowly in the initial 4 d, during which time nitrate concentration slowly decreased while *Fv*/*Fm* increased. When the cells entered the exponential growth phase, *M. aeruginosa* reached maximum growth rate and *Fv*/*Fm* ratio along with the rapid consumption of nitrate, and presented significantly higher μ in 7 mg-N/L treatment than that in 0.5 mg-N/L treatment (Table S3 in Supporting Information; ANOVA, $p < 0.05$).

Compared to nitrate, ammonium played a dual role in *M. aeruginosa* growth. Cell density in the 3 mg-N/L ammonium treatment showed no significant differences from the 7 mg-N/L ammonium treatment during 0–14 d (ANOVA, $p > 0.05$). In the 7 mg-N/L ammonium treatment, significantly lower cell densities and *Fv*/*Fm* were observed than nitrate and urea treatments (ANOVA, $p < 0.05$), implying that *M. aeruginosa* growth was strongly suppressed. This could be attributed to the accumulation of ammonia in cells under high ammonium conditions. Compared to ammonium, the undissociated and uncharged ammonia molecular is lipid soluble, enabling it to easily diffuse across cell membrane. The free internal ammonia exerts toxic effects on algal growth not only by inducing photodamage of photosystem II (Collos and Harrison, 2014), but also by disturbing cytosolic pH because of the production of $H+$ as a byproduct of growth (Britto and Kronzucker, 2002). A previous field

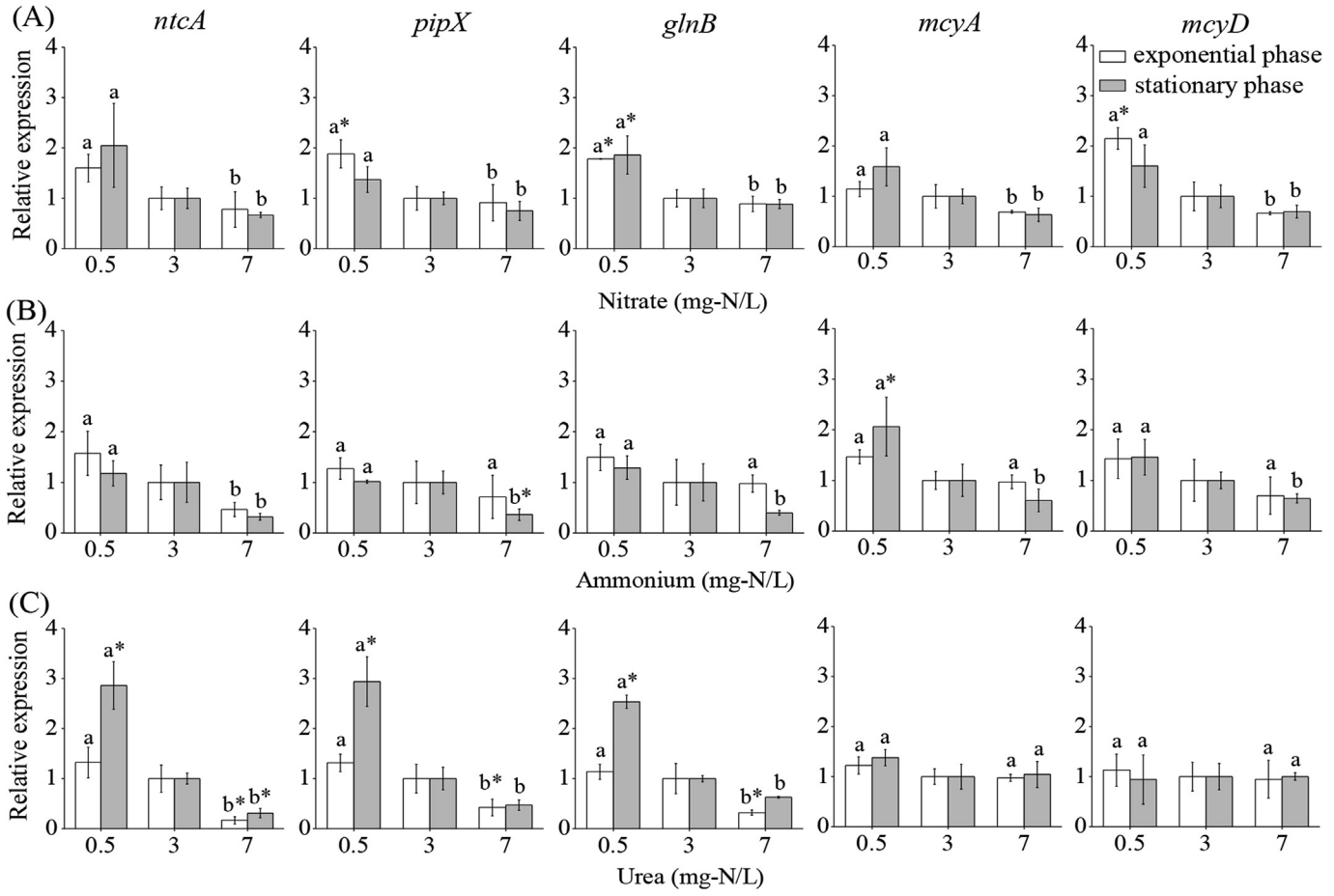

Fig. 6. Transcripts of N metabolism-associated genes (*ntcA*, *pipX*, and *glnB*) and MCs biosynthesis-associated genes (*mcyA* and *mcyD*) under different forms and concentrations of N (A: nitrate treatment; B: ammonium treatment; C: urea treatment). The 3 mg-N/L treatment was used as the control. Different letters indicate statistically distinguishable observations, and asterisk indicates significant difference compared to the control (ANOVA, $p < 0.05$).

study of 33 Chinese lakes confirmed that ammonium is detrimental to algae growth at higher concentrations (Wu et al., 2006). However, ammonium at low concentrations is more easily absorbed by algae than other biologically available forms of N (Berg, 2003). Thus, although cell growth under ammonium treatments experienced a time-lag, the concentrations of ammonium dropped more rapidly than the concentrations of nitrate during initial cultivation due to the faster uptake of ammonium.

In the urea treatments of this study, *M. aeruginosa* growth was stimulated by the increase in N concentration, presenting comparable cell densities to nitrate treatments (Fig. 1). Previous studies have revealed that *M. aeruginosa* can develop well using urea as the sole N source, and even obtain higher growth than using other organic or inorganic nitrogenous compounds (Berman and Chava, 1999; Li et al., 2016). However, Wu et al. (2015) reported that cell growth was restricted by high urea concentration of 3.6 mmol-N/L, and Erratt et al. (2018) also found that under 7 mmol-N/L urea treatment, cell growth could be completely inhibited. In a high urea environment, excessive ammonium is produced by urea hydrolysis and excreted into the medium, creating unfavorable conditions for the growth of *M. aeruginosa* (Sakamoto et al., 1998). Thus, the environmentally relevant urea concentrations (0.5–7 mg-N/L) selected for this study were suitable for *M. aeruginosa* growth. As a reduced N form, urea was rapidly and largely taken by *M. aeruginosa*, with a final efficiency η of nearly 100%. The massive uptake of urea may not only enhance *M. aeruginosa* growth, but also participate in pigment synthesis. As observed by Erratt et al. (2018), cells present higher concentrations of chlorophyll-*a* and phycocyanin under urea treatment than under other N treatments.

4.2. Effects of different nitrogen forms on MCs production and release

In this study, the IMCs showed different responses to the different forms of N sources. In the nitrate treatments, IMCs levels were negatively associated with nitrate concentration (Fig. 4A), and were much lower in the 7 mg-N/L treatment (about half) than in the 0.5 mg-N/L treatment during the exponential phase. This could be explained from three considerations: (i) As hypothesized by Zilliges et al. (2011), under oxidative stress, MCs were covalently bound to the proteins, including several enzymes of the Calvin cycle, phycobiliproteins and two NADPH-dependent reductases, and increased the stability and enhanced the activity of these proteins, thus increasing the fitness of the toxic strains. The cells cultured in 0.5 mg-N/L of nitrate were under oxidative stress, as shown by the decrease in *Fv*/*Fm* and presence of chlorosis, which may trigger the synthesis of MCs to resist the stress. (ii) In cyanobacteria, the NtcA, PipX and P_{II} proteins, together with 2-OG, constitute a complex network that mediates C/N balance, by regulating the activity and expression of transporters and enzymes related to C/N metabolism (Esteves-Ferreira et al., 2018). As Ginn et al. (2010) and Kuniyoshi et al. (2011) showed, NtcA could bind

to the promoter regions of *mcyA*/*D* as well as *mcyE* and *mcyH*, resulting in the up-regulation of *mcy* genes transcription under nitrate limitations, and the binding affinity was further enhanced by the increased 2-oxoglutarate (2-OG) level. This suggested that MCs production responds to the C and N metabolism equilibrium. Our study also showed that the transcripts of genes associated with MCs biosynthesis (*mcyA* and *mcyD*) and N metabolism (*ntcA*, *pipX*, and *glnB*) varied in the same way in all nitrate treatments. Cells grown under 0.5 mg-N/L nitrate treatments were experiencing nutrient stress, and the transcription of *ntcA*, *pipX*, and *glnB* genes were up-regulated. Higher transcripts of NtcA likely increased their bindings to *mcyA*/*D* promoter regions to up-regulate their transcription, potentially resulting in more MCs synthesis. (iii) The changes in MCs production in response to nitrate concentration were mediated by changes in the intracellular availability of amino acids, especially amino acids associated with MCs synthesis. Cells grown under high nitrate concentration performed higher μ, leading to the decrease in protein quotas, as shown by the negative relationship between them (Fig. S2 in Supporting Information), and amino acids quotas. The decline in the intracellular availability of amino acids, especially the content of amino acids associated with MCs synthesis, led to the decrease in IMCs quotas, as shown by the positive relationship between them ($R^2 = 0.645$, $p < 0.001$). In the nitrate treatments, the EMC levels presented no significant differences among the three treatments in the stationary phase. Thus, the increase in nitrate concentration did not affect the release of MCs.

Under the same N concentration, the cells demonstrated higher IMCs levels under ammonium treatment than under nitrate treatment (Fig. 4A). This indicated that the effects of N on MCs production differed among N species, and ammonium might be more bioavailable for MCs synthesis than nitrate. Previous research has similarly reported that the addition of ammonium resulted in higher MCs content than the addition of nitrate (Donald et al., 2011). The IMCs levels were enhanced by the increase in ammonium concentrations. Compared to 0.5 mg-N/L ammonium treatment, IMCs content were significantly higher (Fig. 4) in 7 mg-N/L ammonium treatment. The significantly higher level of MCs synthesis-associated amino acids in cells grown in 7 mg-N/L ammonium treatment might provide sufficient precursors for MCs synthesis and lead to the higher IMCs contents, as shown by the correlations between IMCs levels and the content of amino acids. However, measurements have to be conducted to directly prove if the amino acids are fully utilized for MC production. Furthermore, IMCs contents exhibited a positive relationship with N uptake rate (Fig. S2 in Supporting Information). Results from continuous culture also suggest that IMCs contents are positively modulated by N uptake rate (Downing et al., 2005). The higher N uptake rate in 7 mg-N/L of ammonium treatment might be another clue to explain the higher IMCs levels under high ammonium condition. This may also shed light on the role of N species in IMCs synthesis, since the cells under ammonium treatments presented the highest N uptake rate and IMCs levels than those in nitrate and urea treatments with the same N level. The high IMCs content in 7 mg-N/L ammonium treatment was not accompanied by the up-regulation of transcriptions of *mcyA* and *mcyD* genes. In cyanobacteria, McyD (*mcyD*) mainly participates in the formation of 3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyl-4,6-decadienoic acid (Adda), while McyA (*mcyA*) incorporates serine and alanine into the growing peptide chain (Tillett et al., 2000). Our results showed that, among the amino acid associated with MCs synthesis, the contents of methionine and leuμμcine were significantly higher in 7 mg-N/L ammonium treatments than those in other treatments, while the contents of other amino acid had little difference. As the incorporation of methionine and leucine was performed by McyB (*mcyB*), the higher IMCs content may be possibly attributed to the increase in transcripts of *mcyB* gene, which had to be further investigated.

Although the IMCs levels in 7 mg-N/L ammonium treatment were two times higher than that of 0.5 mg-N/L ammonium treatment, the EMCs levels showed no significant differences between the two treatments. In the ammonium treatments, compared to the control, low (0.5 mg-N/L) N concentration restricted the growth of *M.aeruginosa* with a significantly lower μ, which may be one explanation for the higher EMCs due to their negative correlation (Fig. S2 in Supporting Information). Meanwhile, the decreased *Fv*/*Fm* represented the occurrence of photodamage caused by N-limitation, which may lead to cell death and hence MCs releasing (Fallon and Brock, 1979; Harke and Gobler, 2013). High (7 mg-N/L) N concentration exerted ammonium toxicity associating with photodamage and disturbance of intracellular pH. With the deterioration of growth conditions, massive MCs were released following cell disruption (Drath et al., 2008). It is therefore important for China to be cautious of her current nitrogen management, as the country is taking great efforts to decrease the high level of ammonium in rivers and lakes (Yu et al., 2019).

The IMCs levels and transcripts of *mcyA* and *mcyD* showed no significant differences among the various urea concentrations, although the protein and amino acids quotas, as well as transcripts of *ntcA*, *pipX*, and *glnB* decreased with the increase in N concentration. A previous study covering a large urea concentration range (0.125−250 mg/L) also reported the same results, with intracellular MC-LR content demonstrating no significant differences among treatments (Huang et al., 2014). Therefore, the utilization of urea for MCs synthesis in *M. aeruginosa* is more intricate than the utilization of inorganic N. As an organic form of N, urea hydrolysis not only provides N but also inorganic C to the cells. The additional N and C are not used for MCs synthesis, but are redirected to other key physiological processes holding high N demands, such as pigment synthesis (Erratt et al., 2018; Harke et al., 2016b). In the urea treatments, EMCs levels in 3 and 7 mg-N/L treatments were comparable, while it was significantly higher in 0.5 mg-N/L treatments. This result further demonstrated that 0.5 mg-N/L urea treatment did not favor *M. aeruginosa* growth and led to MCs release.

5. Conclusions

This study presented the physiological effects of the three commonly bioavailable N sources on the regulation of growth and MCs production of *M. aeruginosa*. Cell growth was found to be promoted by the increase in nitrate, ammonium and urea concentration, but was suppressed by high concentrations of ammonium (7 mg-N/L). Cells grown under high concentrations of ammonium (7 mg-N/L) had high levels of amino acids associated with MCs synthesis, which may favor MCs synthesis. Both high and low ammonium concentration increased EMCs levels, but achieved through different underlying mechanisms. This study improved our understanding of the regulating effects of N (forms and concentrations) on the growth and MC synthesis of *M. aeruginosa*, which is beneficial to screening plausible N management strategies to mitigate toxic algal blooms.

Author contributions

Qiuwen Chen conceived the research idea; Min Wang designed and conducted the experiments, and prepared the manuscript; Qiuwen Chen and Wenqing Shi and Arthur Mynett revised the paper; Hanlu Yan and Liuming Hu assisted in sampling and analysis.

Conflicts of interest

The authors declare no conflicts of interest.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.114890.

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