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On the effect of Fe(III) on proliferation of *Microcystis aeruginosa* at high nitrate and low chlorophyll condition

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ABSTRACT Q3

The impact of Fe concentrations on the growth of *Microcystis* (*M.*) *aeruginosa* in aquatic systems under high nitrate and low chlorophyll conditions was studied. The responses of cell density, total and cell chlorophyll-*a* intracellular Fe content and organic elemental composition of *M. aeruginosa* to different concentration gradients of Fe(III) in the solutions were analysed. The results showed that the proliferation speeds of *M. aeruginosa* were: (1) decelerated when the Fe(III) concentration was lower than 50 μg/L in the solutions, (2) promoted and positively related to the increase of Fe(III) concentration from 100 to 500 μg/L in the solutions over the experimental period, and (3) promoted in the early stage but decelerated in later stages by excess adsorption of Fe by cells when the Fe(III) concentration was higher than 500 μg/L in the solutions. The maximum cell density, total and cell chlorophyll-*a* were all observed at 500 μg Fe(III)/L concentration. The organic elemental composition of *M. aeruginosa* was also affected by the concentration of Fe(III) in the solutions, and the molecular formula of *M. aeruginosa* should be expressed as $C_{7–7.5}H_{14}O_{0.8–1.3}N_{3.5–5}$ according to the functions for different Fe(III) concentrations. Cell carbon and oxygen content appeared to increase slightly, while cell nitrogen content appeared to decrease as Fe(III) concentrations increased from 100 to 500 μg/L in the solutions. This was attributed to the competition of photosynthesis and nitrogen adsorption under varying cell Fe content.

Introduction

In recent years, cyanobacterial blooms occurred frequently in several large shallow lakes in China, and *Microcystis aeruginosa* is reported as the most common algal species (Zhou et al., 2013). Most studies have shown that nitrogen and phosphorus, especially ammonium, nitrate and orthophosphate, are main factors for causing algal blooms directly (Baeka et al., 2015; Xu et al., 2010). Recent studies also discovered that some metals which exist in the water may affect the growth of *M. aeruginosa* and cause algal blooms under certain conditions as well (Boyd et al., 2004; Lewitus et al., 2004).

Fe is an essential element for the synthesis of protochlorophyllide, which is the key component for chlorophyll production, and it also plays an important role in algal cells proliferation. Kong et al. (2014) revealed that changes in the concentration of Fe may alter the dominant algae species and consequently lead to the change of the phytoplankton community in the water. In addition, Fe is an auxiliary element for many enzymes in the cell. Fe is also an important

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component in the reductases of nitrate and nitrite, which plays a significant role in the reduction and transformation of nitrate and nitrite in the process of nitrogen fixation (Ahern et al., 2008; Wang et al., 2010). Other interactions between Fe and ammonia may also affect the absorption of nutrients in algal cells (Flynn and Butler, 1986).

The combination of high nitrate and low chlorophyll is a special condition of the aquatic systems, which is regarded as a transient process, especially for urban lakes and rivers. As for an urban lake or river, this condition may be presented for a while due to the intrusions of urban and nonurban pollutions, or the replenishment of treated wastewater. Even though the water quality may be good and the algae density may be low, such states are very conducive to lead to water bloom as nutrients in the water are sufficient. Many studies conducted on lakes and rivers have shown that under high nitrate and low chlorophyll conditions, Fe might play an important role for phytoplankton growth in those water bodies (Boyd et al., 2004; Gervais, 2002; Atsushi et al., 2003).

Iron element mainly exists as Fe(III) in the near surface region according to the iron cycle mechanism undertaken in the lakes, and this is in accordance with the distribution of algae in lake waters. So Fe(III) is the main iron source for algae in the lakes (Song et al., 2011). This paper studies the effect of Fe(III) concentration on the growth of algae under high nitrate and low chlorophyll conditions, and aim to provide a theoretical basis for controlling algal blooms in this kind of aquatic system.

1. Materials and methods

1.1. Medium with high nitrate concentration and different Fe(III) concentrations

The high nitrate nitrogen (NO_3–N) concentration was set as 20 mg/L (Yan et al., 2015) by adjusting the concentration of sodium nitrate (AR, Kemiou, China) in BG-11 medium. Based on the investigations conducted for concentration detection of metal elements in many water bodies distributed in China, the concentration of Fe(III) was detected as 20 to 400 μg/L for most regions, and besides six concentration levels of Fe(III)/L in the medium as 50, 100, 300, 500, 1000, and 2000 μg/L and another control group with concentration of 10 μg Fe(III)/L were employed by adding appropriate amounts of ammonium ferric (AR, Kemiou, China) citrate to the medium.

1.2. M. aeruginosa inoculation and cultivation

The *M. aeruginosa* used in this study was obtained from the Institute of Hydrobiology, Chinese Academy of Science, and its code was FACHB-912. The process of inoculation and cultivation of *M. aeruginosa* were explained as follows.

Firstly, the culture solution of *M. aeruginosa* was concentrated by centrifugation (Avanti J-26XP, Beckman, USA) at a speed of 2000 r/min for 10 min, and the isolated *M. aeruginosa* was washed three times by sterile sodium bicarbonate (AR, Kemiou, China) solution so as to remove extracellular nutrients, especially the Fe ions. Secondly, *M. aeruginosa* was inoculated in the BG-11 medium, but without any Fe sources, for 4 days to exhaust the intracellular Fe in the cells (Muellar, 1985), and then repeated the centrifugation process to remove the Fe released from the cells. Thirdly, the isolated *M. aeruginosa* was added to a series of solutions in 500 mL flat-bottomed bottles containing 200 mL medium with high nitrate concentration at different Fe(III) concentrations. The initial cell density was set as 4×10^4 cells/mL, which are much lower than the eutrophication threshold of 1×10^6 cells/mL, so as to observe the complete process from oligotrophic to eutrophic conditions during the experiment. The pH of all solutions was adjusted with 1 mol/L hydrochloric acid (AR, Kemiou, China) to 7.1 (Souad et al., 2009). Finally, all solutions were placed in an illumination incubator (Memmert I, Germany). The temperature inside the incubator was kept at (25 ± 0.5)°C, and the luminance was set as 3000 lx with a light/dark cycle of 12 hr/12 hr. The solutions under each Fe(III) condition had three replicates. Each bottle was shaken three times every day during the experiment.

1.3. Indexes for detection of M. aeruginosa growth

Cell density was measured every two days by using a flow cytometry machine (BD Accuri C6, USA), and chlorophyll-*a*, Fe content and element composition were measured at the end of the experimental period. The 10 mL solution was taken for chlorophyll-*a* detection, which was centrifuged to obtain *M. aeruginosa* cells, and chlorophyll-*a* were extracted from them by using 90% ethanol (AR, Kemiou, China) at 4°C in a dark environment for 8 hr. Total chlorophyll-*a* was measured using a spectropolarimeter (RC-6 Plus, USA). Chlorophyll-*a* which exists in each cell, named as cell chlorophyll a in this study, was calculated by dividing the total chlorophyll by the number of cells. A 40 mL solution was taken for the detection of Fe content and Franklin method (Franklin et al., 1998) was employed to separate intracellular Fe, extracellular Fe and solution Fe. Intracellular Fe, named as cell Fe content in this study, was digested and detected by using ICP-MS (series 200-ElanDRC-e, PE, USA). The remaining solution was centrifuged to obtain *M. aeruginosa* cells for the analysis of element composition which was implemented by using an organic element analyser (Thermo Fisher, FLASH 2000, USA) according to JY/T017-1996.

1.4. Analysis methods and models

The average specific growth rate was calculated by Eq. (1):

$$\mu = \ln(X_t/X_0)/t \quad (1)$$

where, X_0 (cells/mL) is the initial cell density, and X_t (cells/mL) is the cell density on the day of t at the end of the experimental period, when the increasing rate of cell density comparing to that detected on the day of $t-1$ is smaller than 5% (Wang et al., 2014).

Experimental data were examined by ANOVA and Bonferroni successively for analysing their difference and effectiveness (Significance level α is 0.05). Data were processed using Excel 2007 and were presented as mean ± standard deviation except for the organic element analysis data. The statistical analysis was performed by using SPSS version 18.0 (IBM corp., USA).

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2. Results

2.1. *M. aeruginosa* growth

Fig. 1 shows the colour changes of different solutions on days 0, 7 and 12. From Fig. 1a, we can notice that there is significant difference among those groups, and we can find that the solutions in which Fe(III) concentrations were 10 and 50 μg/L tended to become yellow from light green on day 7, and the colour gradually becomes deeper in the following days. As shown in Fig. 1c, the colour of solutions with 1000 and 2000 μg/L Fe(III) turned yellow from dark green on day 12 and deeper in the following days. These phenomena indicate that cell growth may stop in a short time if the initial Fe(III) concentration in the solution is quite low because it is the necessary nutrient for cell growth. Of course, the growth will eventually be slowed down if the Fe(III) concentration is too high in the solution because it may lead to high Fe content inside the cells.

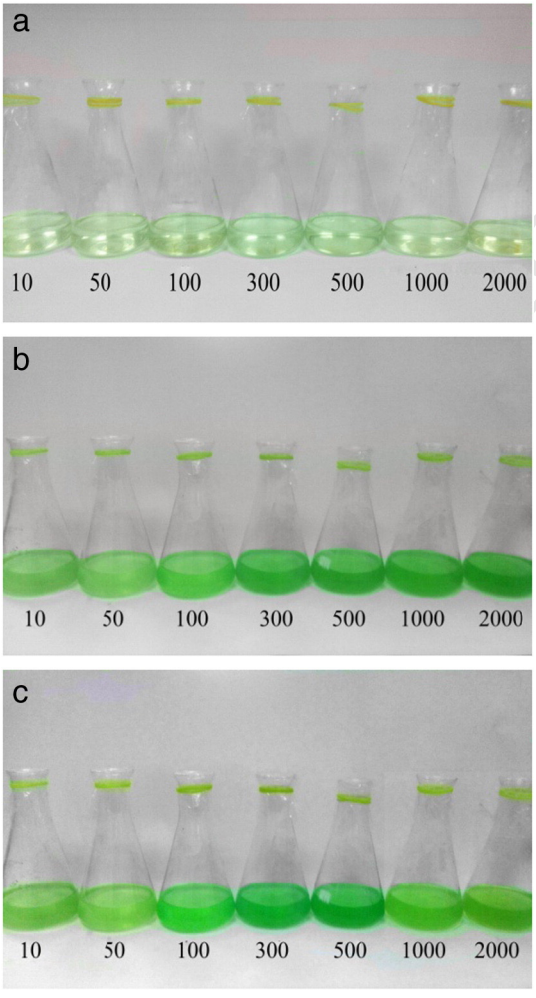

Fig. 1 – Colours of solutions under different Fe(III) concentrations. (a) Day 0, (b) day 7, (c) day 12. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2 shows the changes in cell density under different Fe(III) concentrations, which can explain the phenomena showed in Fig. 1. Compared with the control solution with 10 μg Fe/L concentration, cell proliferations in all the other solutions were significantly greater from the 4th day. Cell densities in the solutions under low Fe(III) concentrations of 10 and 50 μg Fe/L were significantly lower than those under higher concentrations. However, there were also no significant differences in cell density in the solutions under the 1000 and 2000 μg/L concentration levels of Fe(III).

As shown in Fig. 3, X_{max} is used to express the maximum cell density and μ for the average specific growth rate. When the initial concentration is 500 μg Fe/L, the greatest X_{max} was obtained at 3.17×10^6 cells/mL. The X_{max} values for 10, 50, 100, 300, 1000 and 2000 μg/L were 7.4%, 13.2%, 53.6%, 84.1%, 91.6% and 86.5%, respectively, of that for 500 μg Fe/L. The results of μ showed a trend similar with those for X_{max}. In summary, the promoting effect of Fe(III) concentration on the growth of *M. aeruginosa* can be strengthened by increasing the Fe(III) concentration at appropriate concentration levels, and be weakened by increasing the Fe(III) concentration to quite high concentration levels. These findings are similar to Hormesis effect of cell responses to external nutrients, which reveals that cell growth rate shows positive relationship to the nutrient concentration at appropriate concentrations, but in case the concentration is beyond a critical value it shows an opposite or irrelevant relationship (Gong et al., 2009).

2.2. Differences in total and cell chlorophyll-*a*

Fig. 4 shows the total chlorophyll-*a* (T-Chl*a*) and cell chlorophyll-*a* (C-Chl*a*) of *M. aeruginosa* cells extracted from different solutions at the end of the experiment. The results are in accordance with those obtained for cell densities in Fig. 2 and also the growth rates showed in Fig. 3. In the appropriate Fe(III) concentration range of 10–500 μg/L, T-Chl*a* and C-Chl*a* increased with Fe(III) concentrations in the solutions, while in the Fe(III) concentration range of 1000–2000 μg/L, they decreased as the Fe(III) concentration increased. The maximum T-Chl*a* and C-Chl*a* were obtained when the Fe(III) concentration was 500 μg/L. There was almost no difference for T-Chl*a* and C- C-Chl*a* under 1000 and 2000 μg/L of Fe(III) concentrations. Q6

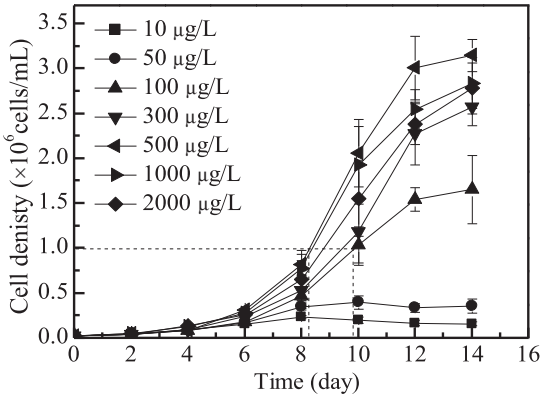

Fig. 2 – Cell densities of *M. aeruginosa* under different Fe(III) concentrations.

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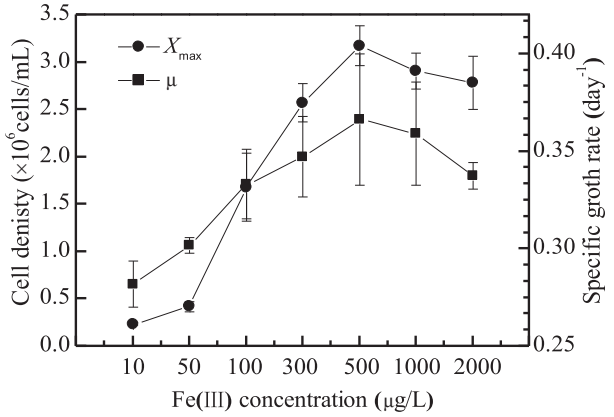

Fig. 3 – Difference in maximum cell density (X_{max}) and average specific growth rate (μ) for different Fe(III) concentrations.

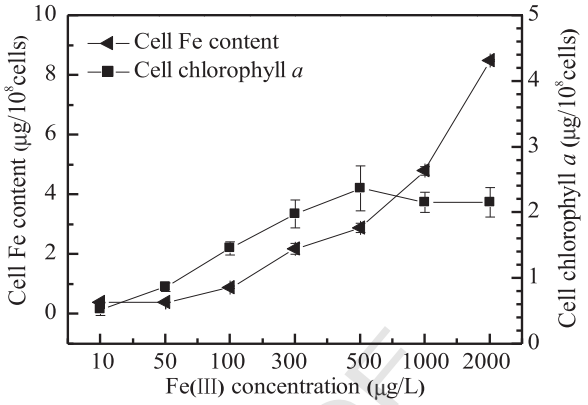

Fig. 5 – Comparison of cell Fe content and cell chlorophyll-*a* under different Fe(III) concentrations.

2.3. *Adsorption of Fe by M. aeruginosa cells*

Fig. 5 shows the results of comparing Fe content inside cells and cell chlorophyll-*a* under different Fe(III) concentrations. When the concentrations of Fe(III) were 10 and 50 μg/L, the value of Fe content inside cells kept almost the same as 0.4 μg/10^8 cells. This indicates that cells maintain a basic Fe content even when Fe is a nutrient insufficient in the surrounding environment. However, as the Fe(III) concentrations increased in the solutions, the Fe content inside cells increased accordingly. When there are sufficient Fe sources in the surrounding environment, the adsorption capacity of Fe by cells is enhanced, thus leading to a higher Fe content inside the cells.

However, comparing with the results for C-Chl*a*, which reflect the growth and proliferation of *M. aeruginosa* cells, high Fe content inside cells does not mean high C-Chl*a*. When the Fe content is deficient, the normal synthesis of chlorophyll is limited, which can affect the photosynthesis in algae cells and can slow down their proliferation as well. That is why the colour of the solutions under low Fe(III) concentrations of 10 and 50 μg/L tended to change from green to yellow on day 7 and to become deeper in the following days (Fig. 1a). When the Fe(III) concentration increased above 50 μg/L, the Fe insufficiency was gradually relieved, and chlorophyll production was promoted. C-Chl*a* was positively related to the cell Fe content, and the colour of solutions with 100, 300 and 500 μg Fe/L concentrations did not appear abnormal until the end of the experiment. Although the Fe content inside the cells increased with Fe(III) concentrations ranging between 500 and 2000 μg/L, the C-Chl*a* decreased. The reason seems to be that excessive absorption of Fe occurred because the concentrations of Fe(III) in the solutions exceed the value required for normal metabolism of cells, and many abnormal processes such as chelating reactions may take place during which toxic substances were produced affecting the growth of cells (Ahern et al., 2007) and then the proliferation speed was decelerated.

2.4. *Organic element composition*

Fig. 6 shows the changes of cell elementary composition under different Fe(III) concentrations. Carbon (C), hydrogen (H), oxygen (O) and nitrogen (N) are the basic organic elements of *M. aeruginosa* cells. The percentage of H content in the cells remained almost unchanged as Fe(III) concentration increased, while that of C, O and N content changed in different

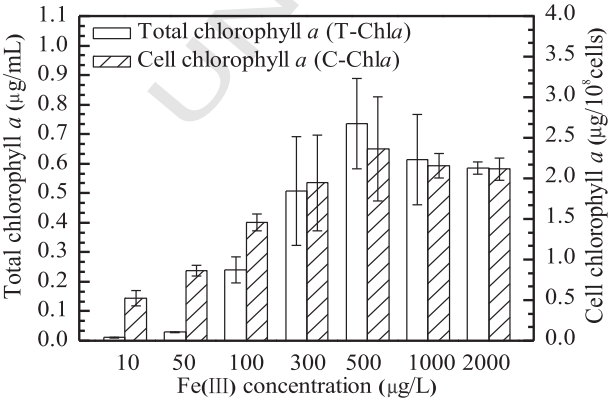

Fig. 4 – Differences in total chlorophyll-*a* and cell chlorophyll-*a* under different initial concentrations of Fe(III).

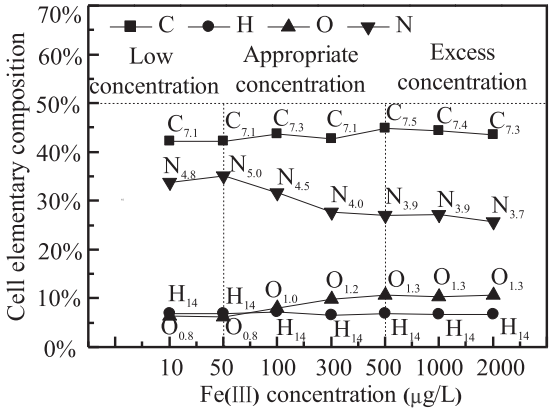

Fig. 6 – Change of cell elementary composition under different Fe(III) concentrations.

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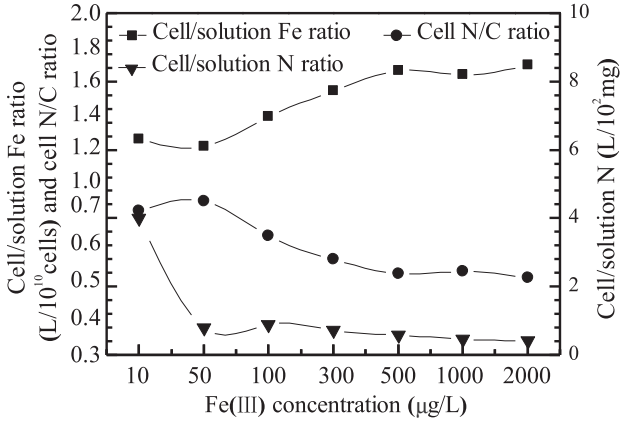

Fig. 7 – Relationship of cell/solution Fe ratio, cell/solution N and cell N/C ratio.

levels. The content percentages of C and O remained stable when the Fe(III) concentrations were 10 and 50 μg/L, but they kept smooth increasing when Fe(III) concentration changed from 50 to 500 μg/L, and then remained stable again when the Fe(III) concentration was more than 500 μg/L. For N, the content percentage increased slightly under Fe(III) concentration from 10 to 50 μg/L, but it decreased sharply when Fe(III) concentration was from 50 to 500 μg/L and then remained stable again when the Fe(III) concentration was from 500 to 1000 μg/L. With the increase of Fe(III) concentration from 1000 to 2000 μg/L, the content percentage of N decreased slightly.

At low Fe(III) concentration levels of 10 and 50 μg/L, the normal growth of *M. aeruginosa* was inhibited because of insufficient Fe intake, and the inhibition of basic metabolic functions, such as photosynthesis and nitrogen adsorption. In this condition, once the Fe(III) concentration increased from 10 to 50 μg/L, the function of nitrogen adsorption was slightly improved because of the high nitrate concentration in solutions. With appropriate Fe(III) concentrations from 100 to 500 μg/L, which can satisfy the need for Fe for normal growth of *M. aeruginosa*, as mentioned above, chlorophyll production and relevant photosynthesis were promoted by the increase of Fe(III) concentrations, which resulted in increased cell C and O contents. In contrast, the cell N content decreased significantly in this range of Fe(III) concentrations, mainly because nitrate reductase and dinitrogenase would be inhibited by the enhancement of the photosynthesis process, which lowered the adsorption capacity of cells to nitrogen. For the quite high Fe(III) concentrations of 1000 and 2000 μg/L, chlorophyll production and photosynthesis were inhibited because of excess intake of Fe, which resulted in decreased cell C content and the stabilization of cell O content. On the contrary, the decrease of cell N content was relieved by the reduction of inhibition on nitrate reductase and dinitrogenase from photosynthesis.

3. Discussion

As a crucial metal element of algae cell, Fe involved in the metabolism process of algae growth, and plays an important role in *M. aeruginosa* growth in aquatic system under high nitrate and low chlorophyll conditions. Based on the above results and analysis, Fe concentration in the solutions had positive relationship with cell Fe content and cell element composition, which were believed to have an impact on several macroscopic indicators of algae growth, such as chlorophyll-*a* and cell density. As shown in Fig. 7, the cell/solution Fe ratio was used to explain the difference of Fe between the extracellular and intracellular of *M. aeruginosa*, and the cell/solution N was used to explain the difference of N (Krivtsov et al., 2005). Besides, cell N/C ratio was used to express the changes of cell N and C due to the competition between photosynthesis and nitrogen adsorption processes (Wang et al., 2013).

It can be concluded from Fig. 7 that the cell/solution N ratio and cell N/C ratio had close relations to the cell/solution Fe ratio. When the cell/solution Fe ratio was quite high which meant that the Fe in solution was deficient and the basic cell Fe content was relatively high but difficult to be sustained, nitrogen adsorption was easy to be promoted along the slight increase of Fe concentration in solution due to the condition of high nitrogen concentration in solution. In this period cell N/C ratio appeared to be slightly increased due to the dual result from (1) the deficiency of cell Fe content during photosynthesis process which led to the decrease of carbon element percentage in cells; and (2) promoted nitrogen adsorption by using limited cell Fe which led to slight increase of nitrogen element percentage in cells. When the cell/solution Fe ratio was below 1×10^{-8}/L, which meant that the Fe in the solution was enough to sustain the basic demand of cell Fe content and make it keep rising, the photosynthesis process was significantly promoted and accordingly the cell chlorophyll content increased rapidly, which led to the decreasing of cell N/C ratio. In addition, the cell/solution N appeared to decrease because of the prior use of cell Fe by photosynthesis process over nitrogen adsorption which was slowed down due to limited Fe support. When the cell/solution Fe ratio was stabilized around 0.5×10^{-8}/L which meant that the balance of Fe in cell and solution was formed and cell Fe content was linear to solution Fe concentration, cell N/C ratio and cell/solution N ratio tended to be stable because of the relative stable element composition of *M. aeruginosa* in spite of the cell Fe content being increased to a high level.

4. Conclusions

The combination of high nitrate and low chlorophyll is a special condition of aquatic systems, especially for urban lakes and rivers, it happens probably because of the intrusion of urban or nonurban pollutants. Under this condition, some trace elements, as well as nitrogen and phosphorus, which are nutrients, are believed to accelerate algae proliferation and to result in eutrophication if their concentrations are in appropriate ranges. This paper studied the impact of Fe on the growth of *M. aeruginosa* in aquatic systems under high nitrate and low chlorophyll condition. The specific findings obtained from this study are as follows.

(1) The concentration of Fe(III) in solutions has a positive impact on the growth of *M. aeruginosa* under the

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condition of high nitrate and low chlorophyll. Low Fe(III) concentration will weaken this positive impact because of intracellular Fe deficiency. The appropriate concentration of Fe(III) is believed to promote growth, and the maximum cell density and chlorophyll-*a* were observed when the Fe(III) concentration was 500 μg/L in the solution. Quite high Fe(III) concentration in solution will eventually result in decelerating growth rate when excess Fe is adsorbed by cells.

(2) The organic element composition of *M. aeruginosa* can be affected by the Fe(III) concentrations in solutions, which means that the molecular formula of *M. aeruginosa* should be different under different concentrations of Fe(III) and it can be expressed as $C_{7–7.5}H_{14}O_{0.8–1.3}N_{3.5–5}$ based on different percentages of various element compositions. Content percentages of carbon and oxygen element appeared to increase slightly, while that of nitrogen element appeared to decrease as the Fe(III) concentrations increased from 100 to 500 μg/L in solutions. This may be attributed to the competition of photosynthesis and nitrogen adsorption at different cell Fe contents caused by different Fe(III) concentrations.

(3) These findings are useful for decision-making on adjusting Fe concentrations, and the critical range of Fe concentrations should be limited to minimize algal blooms in aquatic systems under high nitrate and low chlorophyll condition. Further work should be implemented to study the metabolic mechanisms of Fe in algae cells.

Acknowledgments

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