# The effects of different nitrogen compounds on the growth and microcystin production of *Microcystis aeruginosa*

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## ABSTRACT

The effects of different nitrogen compounds, which include bicine, sodium nitrate, ammonium chloride, glutamic acid and urea, on growth and microcystin (MC) production were studied in batch cultures. The results showed that growth and microcystin production were promoted by bicine, sodium nitrate and ammonium chloride and could not be supported by urea and glutamic acid. It is likely that the cells of *Microcystis aeruginosa* have no urease which can be utilized to decompose urea. Glutamic acid enters the cell and may interrupt the normal metabolic processes. This would inhibit growth and microcystin production, resulting in the death of the organism. Bicine kept the pH of the medium at about 8.5 and offered a preferential environment for growth and microcystin production. Ammonium chloride could be utilized by *Microcystis aeruginosa* indirectly. During the process of assimilation, hydroxy ions were continuously consumed, which would lead to a reduction in pH to less than 7 and retard growth and microcystin production. In the presence of sodium nitrate, the metabolic and biochemical mechanisms, which use sodium nitrate as a source of nutrition during the process of expanding vaccination, were impossible to change in a short time. Thus, sodium nitrate promoted growth and microcystin production.

**Key words** | growth, microcystin production, *Microcystis aeruginosa*, nitrogen compounds

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## INTRODUCTION

The eutrophication of lakes, ponds and reservoirs is caused by an increase in nitrogen and phosphorus, leading to the occurrence of harmful cyanobacterial blooms, which are becoming a serious environmental problem worldwide. Microcystins (MCs) are produced by various cyanobacteria such as *Microcystis, Anabaena, Oscillatoria* and *Nostoc* species, which inhibit eukaryotic protein phosphatases and cause excessive phosphorylation of cytoskeletal filaments and liver failure of animals. Among the toxic cyanobacteria, *Microcystis aeruginosa* (*M. aeruginosa*) is commonly observed in highly eutrophic lakes (Watanabe & Oishi 1985). The MCs produced by *M. aeruginosa* have been the cause of several poisonings of domestic animals and wildlife around the world, and they also pose a health hazard for

humans through the use of water for drinking and recreation (Hoeger *et al.* 2007). Understanding the conditions of MC production by harmful cyanobacterial blooms is of fundamental significance for the control of this environmental problem.

It is well known that nitrogen is not only a main nutrient for cyanobacterial growth but also a key element in the composition of MCs. Furthermore, nitrogen accounts for over 14% of the molecular weight of microcystin-LR (MC-LR). Therefore, many scientists have focused on the effects of nitrogen, especially dissolved inorganic nitrogen (DIN) on MC production using the *M. aeruginosa* species (Vezie *et al.* 2002; Downing *et al.* 2005; Amé & Wunderlin 2005). However, various nitrogenous forms occur in

eutrophic water (Flynn & Butler 1986). Among these nitrogenous forms, urea is ubiquitous in eutrophic water (Berman 1974; Berman *et al.* 1999) and has been demonstrated to provide nitrogen for phytoplankton by many researchers around the world (Berman 1997; Berman *et al.* 1999; Glibert & Terlizzi 1999). Amino acids are available forms of dissolved organic nitrogen (DON) in water (Gardner & Lee 1973; Ittekkot 1988), of which glutamic acid, glycine, alanine and aspartic acid are most abundant (Lytle & Perdue 1981; Ittekkot 1982; Rosa & Nunes 2005; Duan & Bianchi 2007; Gors *et al.* 2007). When the concentration of DIN is low or depleted, urea and amino acids may provide a significant source of available nitrogen for *M. aeruginosa*. Comparative studies on MC production by *M. aeruginosa* have been limited to investigate the effects of inorganic nitrogen. The roles of dissolved organic nitrogen on the growth and production of MC have not been paid much attention.

MC-LR is the most commonly found toxin among many cyanobacterial blooms worldwide (Rinta-Kanto *et al.* 2005) and is one of the most toxic microcystins with an LD50 $\mu g\,kg^{-1}$ of 50 in mice (Rinehart *et al.* 1994). In the present study, a strain of *M. aeruginosa*, the toxin of which was mainly MC-LR, was studied. The effects of different nitrogen compounds on growth and production of MC-LR were investigated. Results indicated that chemical forms of bicine, nitrate and ammonium played important roles in promoting the growth and production of MC-LR by cyanobacteria, which may be important for understanding and practical control of harmful cyanobacterial blooms.

## MATERIALS AND METHOD

### M. aeruginosa

An axenic strain of *M. aeruginosa*, which was isolated from Dianchi Lake in China, was obtained from Institute of Hydrobiology, Chinese Academy of Science.

### Culture medium and culture conditions

*M. aeruginosa* was maintained in BG11 medium (Table 1). Initial pH of the medium was adjusted to 8.5 using $1.0\,mol\,l^{-1}$ hydrochloric acid and sodium hydroxide solutions. The medium and all experimental utensils were kept at 121°C for 20 min in order to sterilize the bacteria. Cultures in exponential phase were concentrated by centrifugation and washed three times with sterile distilled water and then inoculated in the same growth medium, but without a nitrogen source for a week to exhaust the nitrogen in the cells. The *M. aeruginosa* were then added to a series of 2.5-l flat-bottomed bottles which contained 2l medium and were supplemented with $250\,mg\,l^{-1}$ of one of the following nitrogen compounds: bicine, sodium nitrate, ammonium chloride, glutamic acid and urea. Control bottles were incubated with the same algal inocula without nitrogen medium.

**Table 1** | Composition of BG11 medium for *Microcystis aeruginosa**

| Nutrients | Concentration $(g\,l^{-1})$ | Trace metal mix $A_5$ | |
|---|---|---|---|
| $NaNO_3$ | 1.500 | $H_3BO_3$ | 2.86 g |
| $K_2HPO_4$ | 0.04 | $MnCl_2 \cdot 4H_2O$ | 1.81 g |
| $MgSO_4 \cdot 7H_2O$ | 0.075 | $ZnSO_4 \cdot 7H_2O$ | 0.222 g |
| $CaCl_2 \cdot H_2O$ | 0.036 | $CuSO_4 \cdot 5H_2O$ | 0.079 g |
| Citric acid | 0.006 | $NaMoO_4 \cdot 2H_2O$ | 0.390 g |
| Ferric ammonium | 0.006 | $Co(NO_3) \cdot 6H_2O$ | 0.049 g |
| EDTA (dinatrium salt) | 0.001 | Distilled water | 1,000 ml |
| $NaCO_3$ | 0.02 | | |
| Trace metal mix $A_5$ | 1.0 ml | | |
| Distilled water | 919 ml | | |

*pH should be about 8.5 after sterilization.

All samples were run in triplicate bottles at 26°C and a light intensity of 400 ∼ 650 lx with a 12 h photoperiod. All flat-bottomed bottles were shaken by hand three times every day during the maintenance and experimental stages. Sub-samples were taken at predetermined intervals after inoculation during the incubation period to count the cell number and extract the MC.

### Measurement of cell number

After straining with Lugol's iodine solution, the population of *M. aeruginosa* cells was twice counted under a microscope using a haemocytometer. Cell counts were performed to a minimum precision of 20%, or a third cell count was needed. At the same time, the medium was inspected to ascertain whether *M. aeruginosa* had been contaminated by extraneous bacteria.

### Determination of MC within the cells

After inoculation, a 50 ml sample of cyanobacterial culture solution was taken at two-day intervals. The samples were centrifuged at 8,000 r min$^{-1}$ for 10 min and then the supernatant was discarded. The cell pellet was frozen at −20°C for over 24 h, then 20 ml 50% methanol-water solution was added to the cell pellet to extract MC within the cyanobacterial cells. The extraction solution was crushed using an Ultrasonic Cell Disruptor (JY92-2D, SCIENTZ, China) for 30 min and then was put in boiling water for 20 min to extract MC. The solution was centrifuged at 8,000 r min$^{-1}$ for 10 min and the supernatant was filtered through 0.22 μm membranes. The filtration was enriched to 1 ml by mild nitrogen gas with a nitrogen evaporator (KL512, Kanglin Co., China) and measured for MC on HPLC. The MC content in the cells was calculated from the ratio of the concentration of MC in the extraction solution (mg l$^{-1}$) to the cells in the sub-samples (cells ml$^{-1}$). The data presented here were the average values of three parallel samples with standard deviation.

Standard MC-RR and MC-LR bought from Sigma (98% purity) and samples of MC-RR and MC-LR were analysed by HPLC (Hitachi L-2000) with a reverse ZORBAX SB-C18 column (4.6 mm × 250 mm, Agilent Co., USA) and a diode array detector at 238 nm. The mobile phase was 35% (v/v) acetonitrile-water solution containing 0.05% (v/v) of frozen acetic acid. The flow rate was 1.0 ml min$^{-1}$ and the injection amount was 20 μl.

## RESULTS

### Analysis of MC-RR and MC-LR

Microcystins were identified by their characteristic absorption spectra (238 nm) and retention times and quantified by HPLC analysis. Figure 1(a) shows the HPLC profile of standard solution of 5 mg l$^{-1}$ of MC-RR and MC-LR. Peaks of MC-RR and MC-LR appeared at 4.33 and 6.76 min, respectively. The wavelength of 238 nm was the maximum absorbance of MC-RR and MC-LR, which agreed with the literature (Moollan *et al.* 1996) and was used for the detection of MC on HPLC in this experiment. Good linear relationships between the peak area and the standard concentrations of MC-RR and MC-LR were obtained at a wavelength of 238 nm ($R^2 = 0.998$ and 0.999). Figure 1(b) shows the HPLC profile of MC in the cells of *M. aeruginosa*. It can be observed that the MC in the cells was MC-LR and that the *M. aeruginosa* produced MC-LR but no MC-RR.

### Extraction of MC

The extraction of MC from the cells of *M. aeruginosa* was investigated by using different concentrations of methanol solution containing 100% methanol, 80% methanol, 50% methanol, 10% methanol and 100% water. Figure 2 indicates that the extraction efficiency of MC-LR within cells using 50% methanol was slightly higher than those using other methanol solutions and that of using 100% methanol was worst. Lawton & Edwards (2001) recommended 50–80% methanol as the optimal extraction solution. In the present study, 50% methanol was used to extract MC.

### The effects of different nitrogen compounds on growth of *M. aeruginosa*

In natural water, all kinds of nitrogenous compound exist at the same time. *M. aeruginosa* might assimilate these compounds as nitrogen sources to support its growth. As shown in Figure 3, different nitrogen compounds had
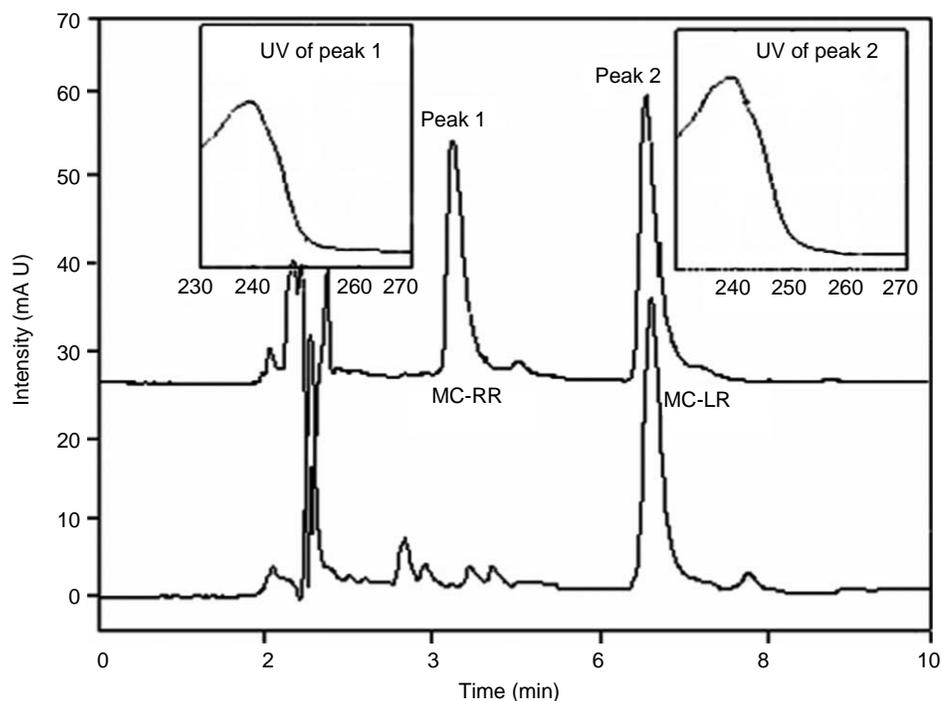
**Figure 1** │ The HPLC profiles for (a) standard MC-RR (RT = 4.33 min) and MC-LR (RT = 6.76 min) and (b) the profiles for MC-LR in the cells of *Microcystis aeruginosa*. Concentrations of standard MC-RR and LR are 5 mg l$^{-1}$, respectively.

different effects on the growth of *M. aeruginosa*. It was shown that *M. aeruginosa* could grow well in a medium containing sodium nitrate, bicine and ammonium chloride, respectively, but could not grow in urea. However, in the presence of glutamic acid, *M. aeruginosa* grew faster than with other nitrogen compounds until 96 h and then became

yellow. Figure 3 indicates that the growth of *M. aeruginosa* in the medium containing sodium nitrate, bicine and ammonium chloride reached exponential phases on the fifth day and late-exponential phases on the 14th day and their growth was a typical example of batch culture growth. Cells of *M. aeruginosa* grown on bicine, sodium nitrate and
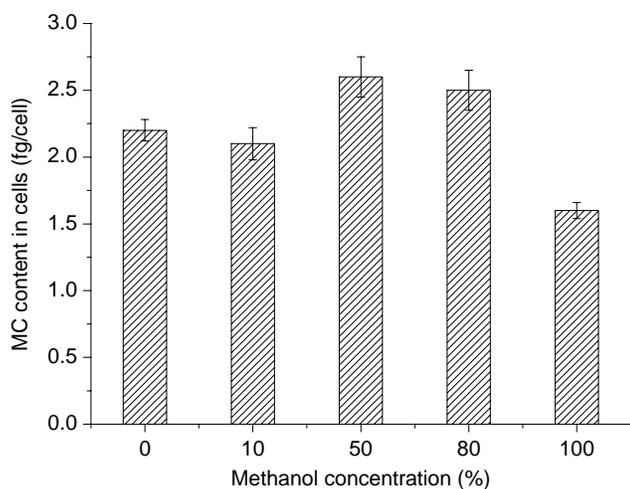


**Figure 2** │ Extraction of MC-LR from *Microcystis aeruginosa* using different concentrations of methanol.
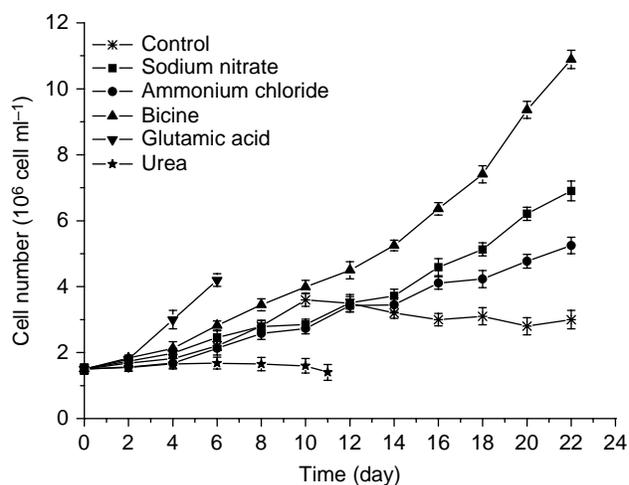


**Figure 3** │ Effects of different nitrogen compounds on the growth of *Microcystis aeruginosa*.

ammonium chloride attained maximum cell numbers of $10.89 \times 10^6$, $6.90 \times 10^6$ and $5.25 \times 10^6$ cells ml$^{-1}$, respectively. Correspondingly, in the presence of glutamic acid, the maximum cell number was up to $3.00 \times 10^6$ cells ml$^{-1}$ at 96 h and exceeded the cell number with bicine. However, growth ceased after 96 h and the appearance of the culture became yellow and turbid.

According to these results, the order of growth promotion of the five different nitrogen compounds was as follows: bicine > sodium nitrate > ammonium chloride > glutamic acid > urea.

## The effects of different nitrogen compounds on MC production of M. aeruginosa

This study has demonstrated that M. aeruginosa can take up bicine, sodium nitrate and ammonium chloride to support its growth, but cannot utilize glutamic acid and urea. However, whether those nitrogen compounds could promote microcystin production was more important. Figure 4 shows the microcystin content with different compounds as the nitrogen source. It is noticeable that the MC content in the presence of bicine, sodium nitrate and ammonium chloride gradually increased up to a maximum on the 10th day from the late-lag phase to the exponential phase; moreover, the MC content declined in the late-exponential phase as shown in Figure 4. This result agreed with the literature (Yan et al. 2004; Amé & Wunderlin 2005). Figure 4 also shows that bicine promoted higher
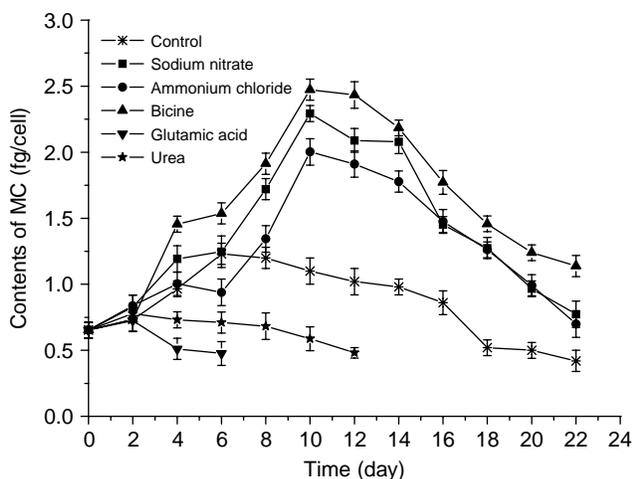


**Figure 4** | Effects of different nitrogen compounds on MC production of Microcystis aeruginosa.

production of MC-LR than sodium nitrate and ammonium chloride. The maximum content of MC-LR in bicine reached 2.47 femtogram/cell (fg/cell) on the 10th day. The MC production of sodium nitrate and ammonium chloride ranked in second and third place with 2.29 fg/cell and 2.00 fg/cell, respectively.

As shown in Figure 4, glutamic acid did not promote the production of MC-LR. Here, maximum MC production was 0.73 fg/cell, lower than that of the control (1.1 fg/cell). The medium containing urea did not increase the production of MC-LR (Figure 4). Its MC production was always lower than that of the control throughout the batch experiments.

Figure 4 shows that the order of nitrogen compounds in terms of their effect on MC production was as follows: bicine > sodium nitrate > ammonium chloride > urea > glutamic acid; similar to that of growth. Our results for the contrasting roles of the five compounds tested in the promotion of growth and the promotion of MC-LR production indicated that the MC production was correlated to the growth of the cyanobacterium.

## DISCUSSION

Cyanobacterial toxin production has been reported to be affected by various environmental factors of which the physical, chemical and biological parameters are most often investigated. We, therefore, focused our study on the effect of different nitrogen compounds on growth and microcystin production in M. aeruginosa, one of the bloom-forming toxic cyanobacteria isolated from Dianchi Lake, southwestern China. Our results showed that microcystin-LR was the major toxin in this strain and 50% methanol had better extraction efficiency. The present study also clearly demonstrated that bicine, sodium nitrate and ammonium chloride could be used as the sole nitrogen source to support the growth of M. aeruginosa and promote MC production. M. aeruginosa could not utilize glutamic acid and urea to support natural and sustaining growth or to stimulate MC production.

Growth and MC production were both inhibited by urea, which may be attributed to the lack of urease in the cells of M. aeruginosa. Urease is the key determinant of

whether the phytoplankton can use urea as a nitrogen source (Amé & Wunderlin 2005). This demonstrated that urea, which is imported into natural water by human activity, does not directly stimulate the growth of *M. aeruginosa*; on the contrary, it could inhibit the growth. However, due to the complexity of the natural water, urea is probably decomposed to ammonia by bacteria and microorganisms, leading to the outbreak of the cyanobacterial bloom. It has been reported that cyanobacteria *Microcystis* and *Synechococcus* could grow well with urea as a nitrogen source (Berman & Chava 1999). However, that experiment was not operated in axenic conditions. Urea was converted into other nitrogen sources by bacteria in the medium. In this condition, the nitrogen source that was utilized by *M. aeruginosa* was not urea. Therefore, the results of the Berman & Chava (1999) study were in opposition to our results.

Glutamic acid was not utilized as a nitrogen source to support the growth of *M. aeruginosa*, although biomass of *M. aeruginosa* was quickly promoted in the first four days. But the promotion was unusual. It has been reported that glutamic acid is crucial in the processes of amino acid uptake, metabolism and transformation within the algal cells, and leucine, tryptophan, valine and aspartic acid were transformed through the metabolism of glutamic acid. In normal algal cells, the concentration of glutamic acid lies within a certain range. When *M. aeruginosa* was poured into the medium containing glutamic acid, it took up a small amount of glutamic acid and its growth was rapidly promoted at the beginning of incubation. However, with substantial absorption of glutamic acid, excessive amounts accumulated in the cells and exceeded the normal range, leading to the destruction of the normal amino acid cycle and influencing the normal physiological process of metabolism. Therefore, *M. aeruginosa* rapidly became yellow and died.

The pH value was also an important factor for growth and MC production. The present study measured the pH of the medium in the presence of bicine, sodium nitrate and ammonium chloride. As shown in Figure 5, during the process of incubation, the pH of the medium containing bicine was in the range 8.2 ~ 8.5, with sodium nitrate the pH was between 8.5 and 9.5, and with ammonium chloride the pH was below 7.

A suitable pH for the growth of *M. aeruginosa* is about 8.5, and acidic conditions are detrimental to growth. It has
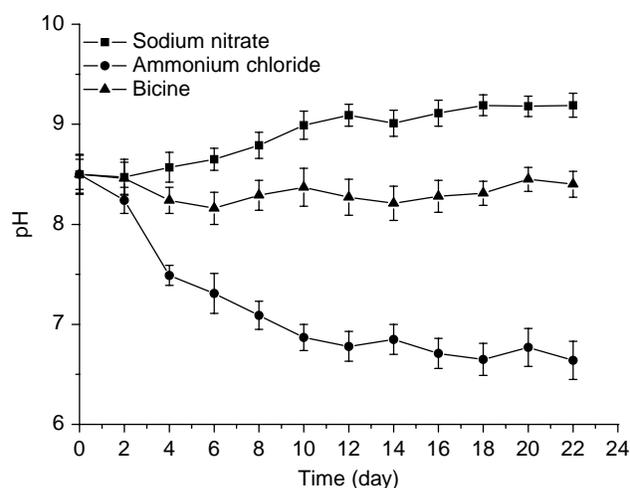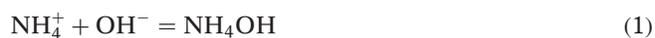


**Figure 5** | pH value of the medium in the presence of sodium nitrate, ammonium chloride and bicine.
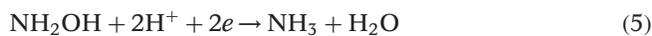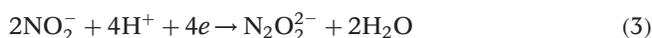
been reported that algae can directly absorb $NH_4^+$ when ammonium chloride exists in the medium. However, ammonium is vulnerable to entering into the cells with the non-dissociation of ammonium hydroxide (Liu 1999), as shown in Equation (1).

$$NH_4^+ + OH^- = NH_4OH \tag{1}$$

The hydroxy ion is continuously consumed in this process, which causes the pH of the medium to decrease. Moreover, pH was lower with a higher concentration of ammonium chloride in the medium. It was possible that pH, which declined during the growth of algae, inhibited the growth of *M. aeruginosa*. In the present study, the concentration of ammonium chloride was $250\,mg\,l^{-1}$. Therefore, the growth and MC production was obviously inhibited. Although it has been reported that MC content would be promoted at lower pH ($<7$) (Amé & Wunderlin 2005), the growth and MC content were largely influenced by low pH. The inhibitory action was greater than the promoting action.

Before nitrate nitrogen is assimilated to synthesize protein and other metabolites as raw materials, it must be first transformed into ammonia (Liu 1999). The four steps are shown in the following Equations (2)–(5).

$$NO_3^- + 2H^+ + 2e \rightarrow NO^{2-} + H_2O \tag{2}$$

$$2NO_2^- + 4H^+ + 4e \rightarrow N_2O_2^{2-} + 2H_2O \tag{3}$$

$$N_2O_2^{2-} + 6H^+ + 6e \rightarrow 2NH_2OH \tag{4}$$

$$NH_2OH + 2H^+ + 2e \rightarrow NH_3 + H_2O \tag{5}$$

Hydrogen ions are continuously consumed during the process of reduction, which increases the pH causing the medium to become alkaline. In the present study, the pH of the medium was up to about 9.5, and high pH would affect the growth of *M. aeruginosa*. However, the *M. aeruginosa* was cultured in the medium containing nitrate during the process of expanding vaccination. Although the nitrogen compounds within the cells were depleted through the process of pre-incubation, the metabolic and biochemical mechanisms, which use sodium nitrate as a source of nutrition, were impossible to change in such a short time. Therefore, nitrate promoted growth and MC production.

Bicine, chemical name N,N-dihydroxyethyl glycine, is a ramification of glycine. Furthermore, it is a buffer and is often used as a biological buffer because the molecule contains two hydroxyls. In this study, bicine was not only a nitrogen source, but also played the role of buffer. The pH of the medium remained at about 8.5, which provided a good growth environment for *M. aeruginosa* and thus promoted the greatest degree of growth and MC production. The bicine supported more rapid growth and a higher content of MC-LR within the cells of *M. aeruginosa*, which is in agreement with the findings that the production of MC was controlled by environmental effects on the rate of cell division of *M. aeruginosa* (Orr & Jones 1998).

Rao *et al.* (1996) found that MA medium containing bicine and nitrate as nitrogen sources supported the best growth of *M. aeruginosa* among the five media tested. Our results confirmed that the nitrogen chemicals of bicine, nitrate and ammonia were important in the rapid growth and MC production of *M. aeruginosa*. They are good nitrogen sources for *M. aeruginosa*. Therefore, they can stimulate the cyanobacterial bloom with a high content of MC. Bicine, an amino acid, promoted the greatest growth and MC production among the tested nitrogen compounds. This demonstrated that amino acids are directly involved in the metabolism of algae and even promoted its growth and

toxin production. Normal physiological metabolism of this cyanobacterium might be disrupted by glutamic acid, which was responsible for the severe inhibition of growth and MC production. This phenomenon requires further study.

The data reported here constitute a basis for investigation into the different nitrogen compounds that correlate with increased MC production rates and suggest that amino acid assimilation in natural water may be involved in the regulation of MC production. Such information might shed light on the nature of the mechanism of up-modulation of MC.

## CONCLUSIONS

(1) Bicine, sodium nitrate and ammonium chloride promoted growth and microcystin production to different degrees. Urea could not support growth and microcystin production. Although cell division was accelerated by glutamic acid at the beginning of the incubation period, growth and microcystin production was not supported.

(2) It is likely that the cells of *M. aeruginosa* do not contain urease which can be utilized to decompose urea. Therefore, urea was not used as a nitrogen source by *M. aeruginosa*. Glutamic acid enters the cells of *M. aeruginosa* and might interrupt the normal metabolic processes. This would inhibit growth and microcystin production, and cause the death of the organism.

(3) Bicine, which is a buffer solution, kept the pH of the medium at about 8.5 and offered a preferential environment for growth and microcystin production. Ammonium chloride could be utilized by *M. aeruginosa* indirectly. During the process of assimilation, hydroxy ions were continuously consumed, which would cause the pH to drop to less than 7, retarding growth and microcystin production.

(4) After sodium nitrate is transformed to ammonia, it can be utilized by *M. aeruginosa*. Hydrogen ions are consumed in the process and result in increased pH. However, the metabolic and biochemical mechanisms, which use sodium nitrate as a source of nutrition during the process of expanding vaccination, were

impossible to change in such a short time. Thus, sodium nitrate promoted growth and microcystin production.

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