【SIV-4】

Microcystin Production by *Microcystis* sp. under N or P Limitation

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Abstract

The production of microcystins from *Microcystis aeruginosa* was investigated in a P-limited continuous culture and a batch culture. The microcystin content of *M. aeruginosa* was higher at a lower μ, whereas the microcystin (MC)-producing rate was linearly proportional to μ. The ratios of the MC-producing rate to the C-fixation rate were higher at a lower μ. Consequently, increases in the microcystin content per dry weight along with the production of the more toxic form, MC-LR, were both observed under more P-limited conditions. The microcystin content of *M. aeruginosa* exhibited a high correlation with the total N content regardless of N-fixed or P-fixed culture. The microcystin concentration was investigated from spring to autumn in 1999 in the Daechung Reservoir, Korea. The dominant species in the algal blooming season was *Microcystis*. When the microcystin concentration exceeded about 100 ng l^{-1}, the ratio of particulate to dissolved total nitrogen (TN) or total phosphorus (TP) interestingly converged at a value of 0.6. The microcystin concentration was lower than 50 ng l^{-1} at a particulate N:P ratio below 8, whereas the microcystin concentration varied quite substantially from 50 ng l^{-1} to 250 ng l^{-1} at a particulate N:P ratio > 8.

Introduction

The bloom of cyanobacterium, *Microcystis aeruginosa*, is a ubiquitous phenomenon in eutrophic lakes and reservoirs in many countries of the world. Many strains of *Microcystis* are known to produce cyanobacterial hepatotoxins called microcystins. These toxins, soluble peptides, are lethal to many kinds of aquatic organisms and damages zooplankton, fish, and the liver of higher animals.

The effects of N and P on the toxin production by cyanobacteria are highly variable (Orr and Jones, 1998; Oh *et al.*, 2001). Batch cultured *M. aeruginosa* decrease in toxicity when N and inorganic C are removed from the medium (Codd and Poon, 1988). The concentration of microcystin in *Anabaena* increases with P (Rapala *et al.*, 1997). Toxin production by *Oscillatoria agardhii* depends on a low-level concentration of P (0.1 to 0.4 mg of P per liter) and higher concentrations have no additional effect (Sivonen, 1990). It was recently reported that the net microcystin production rate decreases as the specific cell division rate decreases in N-limited *M. aeruginosa* cultures (Orr and Jones, 1998). However, information about

microcystin production related to the nutrient status in cells or in ambient circumstances is still insufficient.

In this study, *M. aeruginosa* was cultured in a chemostat to produce a culture in a steady state and clarify the toxin production relative to the growth of *M. aeruginosa* and its nutrient status (with a specific focus on P limitation) both of which seem to be important in eutrophic water. The relationship between the microcystin content of *M. aeruginosa* and the cellular N or P contents was also analyzed with diverse medium N:P ratios in a batch culture. In the field, the microcystin concentrations were analyzed in water and algal samples taken every week during the period of algal bloom and compared with the changes of N and P concentration.

Materials and Methods

Microcystis aeruginosa UTEX 2388, obtained from the Culture Collection of Algae at the University of Texas at Austin, was investigated in a P-limited chemostat at dilution rates ranging from 0.1 to 0.8 d^{-1} at 28℃ using a modified SW medium (Smith and Wiedeman, 1964). The modification included replacing the Tris buffer with HEPES and adjusting the pH to 8.0. The P concentration was reduced to 6 μM. Illumination was continuous, provided by cool white fluorescent lamps at an average light intensity of 160 μE m^{-2} s^{-1} inside the culture vessel. When the culture reached a steady state, aliquots were used to measure the photosynthesis, cellular components, and microcystins. A 500-ml portion of the culture was centrifuged (Sorvall RC5C) for 10 min at 15,000 × g. The supernatant was used for the analysis of any residual P. At all steady states, no residual P was detectable. Cell pellets were then washed by centrifugation and stored at -65℃ for further analysis.

M. aeruginosa was cultured in batch cultures in 1000-ml flasks with 300 ml of a modified SW medium. The medium N:P atomic ratios were set to 1:1, 5:1, 16:1, 50:1 and 100:1 by adjusting the amount of K_2HPO_4 and $NaNO_3$ under N-fixed (71.4 μmol N l^{-1}) and P-fixed (6.5 μmol P l^{-1}) conditions. 71.4 μmol N l^{-1} and 6.5 μmol P l^{-1} were considered as equivalent to 1 mg N l^{-1} and 0.2 mg P l^{-1}, respectively, which is normal in eutrophic waters. The flasks were incubated with continuous shaking (100 rev min^{-1}), a constant temperature of 30℃, and a light intensity of 150 μE m^{-2} s^{-1} using continuous cool white fluorescent lamps.

In the field experiments, the sampling site was located on the shore in the vicinity of the dam in the Daechung Reservoir. The sampling was conducted weekly from the same site from April 27 to October 12, 1999. The samples for water analysis were collected at a depth of 0-0.1 m using a Van Dorn water sampler (WILDCO Instruments) and stored in 20-L polyethylene bottles at 4℃ until the laboratory analysis. The samples for plankton identification and enumeration were preserved in Lugol's solution.

A 500-ml portion of each water sample was centrifuged for 10 min at 15,000 × g. The

supernatant was then used to analyze the level of dissolved N and P. The pellets including any particulate material were washed with distilled water, followed by centrifugation and stored at -65℃ for further analysis. The particulate C was determined using a total organic carbon analyzer (Shimadzu, 5000A). The total N and P were determined after persulfate oxidation to nitrate and orthophosphate, respectively. The nitrate was determined using a Szechrome NB reagent and the orthophosphate using the phosphomolybdate method (Lee *et al.*, 2000).

The purification and analysis of the microcystins were carried out using the method developed by Harada *et al.* (1988). The protein phosphatase (PP) activity was determined with some modifications as previously described by Lambert *et al.* (1994).

Results and Discussion

1. Microcystin production in a P-limited chemostat

The microcystin (MC)-producing rate, derived from the multiplying the microcystin content by μ, increased linearly with μ, while the total microcystin content per unit of dry weight decreased with μ (Fig. 1). The maximum MC-producing rate was about 444 g g^{-1} d^{-1} at μ = 0.8 d^{-1}. The MC-producing rate of the cells increased about 4.0-fold when μ increased from 0.1 to 0.8 d^{-1}, while the microcystin content decreased to 555 g g^{-1} with μ, as a half of 1,118 g g^{-1} at μ = 0.1 d^{-1}. That is, the microcystin contents of the cells at a higher μ were low, whilst the MC-producing rate at a higher μ was higher due to a higher μ.

There have been several reports on the effect of P on microcystin production by cyanobacteria. Watanabe and Oishi (1985) stated that P-deficient cells have a slightly lower toxicity, yet no difference can be seen between cells grown in 1/10 and 1/20 P conditions. Utkilen and Gjølme (1995) reported that phosphate-limited conditions have no effect on the toxin production of *M. aeruginosa*, whereas iron-limited conditions do. Recently, Sivonen (1990) reported that the toxin production of *Oscillatoria agardhii* depends on a low-level concentration of P (0.1 to 0.4 mg of P per liter) and higher concentrations have no additional effect. Accordingly, this indicates the possibility of an increase of toxicity in P-limited conditions. Therefore, it would seem that the MC-producing rate in a water system is determined by μ of *M. aeruginosa* that in turn is determined by the concentration of P in the water body.

2. Relationship between photosynthesis and microcystin production

MC-LR/MC-RR and C-fixation rate/MC-producing rate were plotted as a function of the N:P atomic ratio of the steady-state cells of *M. aeruginosa* in Fig. 2. When the N:P atomic ratio was high (severe P-limited condition), the ratio of MC-LR to MC-RR increased. That is,

the degree of the P limitation affected both the microcystin content of *M. aeruginosa* and the type of microcystin produced. Unfortunately, the toxicity of MC-LR is over 4 times stronger than that of MC-RR judging from the data of the LD_{50} value with mice (Kotak *et al.*, 1995).

There was a tendency of an increase in the MC-producing rate/C-fixation rate in P-limited conditions. The ratios of the MC-producing rate to the C-fixation rate were higher at a lower μ. Thus, the growth of *M. aeruginosa* was reduced under P limitation due to a low C-fixation rate, whereas the microcystin content was higher.

These results strongly suggest that P is an important factor in the control of both the production of microcystin and the type of microcystin produced. In particular, in the P-limited cells of *M. aeruginosa*, there were increasing tendencies for both the microcystin content and the more toxic MC-LR compared to MC-RR. However, in the culture of *M. aeruginosa*, the MC-producing rate was determined by the concentration of P in the water body. Accordingly, these facts should be considered when managing and restoring the water quality in eutrophic lakes where MC-producing cyanobacterial bloom occurs frequently.

3. Microcystin contents in N or P-fixed culture

The microcystin content of *M. aeruginosa* was slightly, negatively correlated with the total P content in the N-fixed culture ($Y = -253.7X + 2041$, $r^2 = 0.165$) (data not shown), yet highly correlated with the total N content regardless of an N-fixed or P-fixed culture ($Y = 347.2X - 496.9$, $r^2 = 0.586$, $P < 0.01$) (Fig. 3). Consequently, the microcystin content of *M. aeruginosa* is highly correlated with the total N content irrespective of an N-fixed or P-fixed culture.

The microcystin content of *M. aeruginosa* was slightly, negatively correlated with the P content under N-fixed conditions. In a similar report, Wicks and Thiel (1990) reported that microcystin concentrations correlate negatively with *ortho*-P in floating scums of *M. aeruginosa*. Recently, Oh *et al.* (2000) also showed that the microcystin content of *M. aeruginosa* increases in the steady-state cells under P-limited conditions.

It has also been previously demonstrated that there is a remarkable decrease in the toxicity of *M. aeruginosa* when grown with a reduced nitrate concentration (Watanabe and Oishi, 1985; Codd and Poon, 1988). Recently, Orr and Jones (1998) reported that the microcystin content of *M. aeruginosa* is two- to three-fold higher during non-N-limited long-phase growth than during the late stationary phase.

4. Relationship between microcystin concentrations and nutrients

The ratio of particulate to dissolved TP or TN was expressed as a function of the microcystin concentration in water (Fig. 4). In the case of TP, the ratio of particulate to dissolved TP decreased dramatically with a microcystin concentration below about 100 ng l^{-1} microcystin. When the microcystin concentration exceeded about 100 ng l^{-1}, the ratio of

particulate to dissolved TP interestingly converged at a value of 0.6.

In contrast, the ratio of particulate to dissolved TN increased with a microcystin concentration below about 100 ng l^{-1} microcystin. When the microcystin concentration exceeded about 100 ng l^{-1}, the ratio of particulate to dissolved TN converged at a value of 0.6. Therefore, it would appear that the ratio of particulate to dissolved form at 0.6 is the threshold value for determining the microcystin concentration. In other words, the microcystin concentration was lower than 100 ng l^{-1} when the ratio of particulate to dissolved TP was high, while the particulate to dissolved TN was low.

Rapala and Sivonen (1998) also suggested that the concentrations of dissolved inorganic N and P may regulate the species and strain composition and, hence, the toxicity of a bloom. Accordingly, since a chemical analysis of N and P in water can provide information on the presence of the cyanobacterial toxin, a simple estimate of the existence of microcystins can be based on an analysis of the ratio of particulate to the dissolved form of N and P.

The amount of toxin in the particulate material correlated positively with the cyanobacteria biomass as well as with the total nitrogen and total phosphorus concentrations in the water (Lahti *et al.*, 1997). In other words, the level of the microcystin concentration was also determined to some extent by the particulate N:P ratio.

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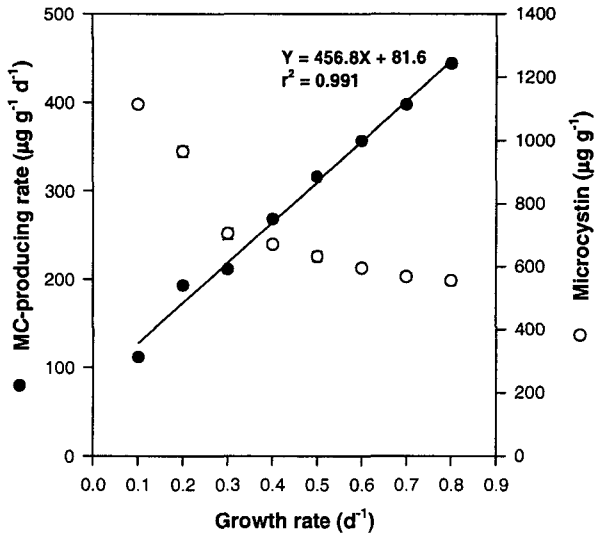

Fig. 1. Microcystin (MC)-producing rate and microcystin content of *Microcystis aeruginosa* at each growth rate in a P-limited chemostat. Error bars indicate SD (n = 3).

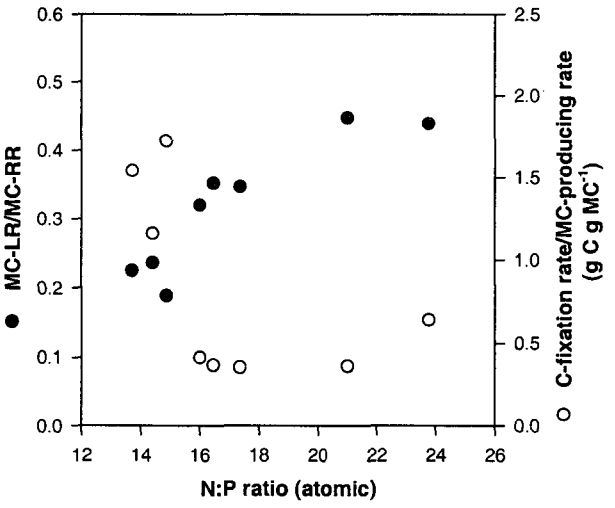

Fig. 2. Relationship between the N:P atomic ratios and the microcystin (MC)-LR/MC-RR or C-fixation rate/MC-producing rate of *Microcystis aeruginosa*.

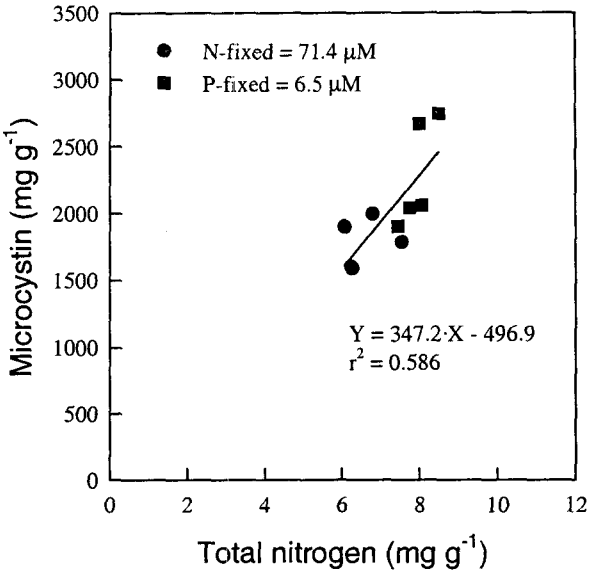

Fig. 3. Relationship between total N and microcystin content of *Microcystis aeruginosa* grown for 7 d under N-fixed and P-fixed conditions.

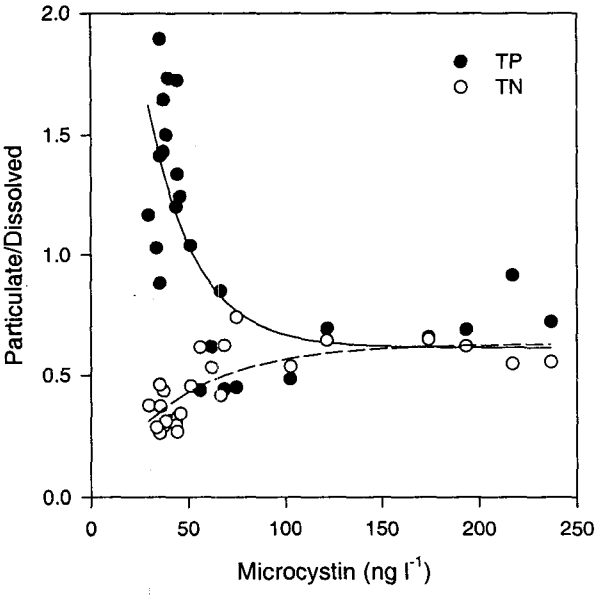

Fig. 4. Relationship between microcystin concentration and particulate to dissolved total phosphorus (TP) and total nitrogen (TN) in Daechung Reservoir.