Microcystin Production by *Microcystis aeruginosa* in a Phosphorus-Limited Chemostat

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The production of microcystins (MC) from *Microcystis aeruginosa* UTEX 2388 was investigated in a P-limited continuous culture. MC (MC-LR, MC-RR, and MC-YR) from lyophilized *M. aeruginosa* were extracted with 5% acetic acid, purified by a Sep-Pak C_{18} cartridge, and then analyzed by high-performance liquid chromatography with a UV detector and Nucleosil C_{18} reverse-phase column. The specific growth rate (μ) of *M. aeruginosa* was within the range of 0.1 to 0.8/day at 28°C by using a medium modified from the cellular P content under a P limitation. The N/P atomic ratio of steady-state cells in a P-limited medium varied from 24 to 15 with an increasing μ. The MC-LR and MC-RR contents on a dry weight basis were highest at μ of 0.1/day at 339 and 774 μg g^{-1}, respectively, while MC-YR was not detected. The MC content of *M. aeruginosa* was higher at a lower μ, whereas the MC-producing rate was linearly proportional to μ. The C fixation rate at an ambient irradiance (160 microeinsteins m^{-2} s^{-1}) increased with μ. The ratios of the MC-producing rate to the C fixation rate were higher at a lower μ. Accordingly, the growth of *M. aeruginosa* was reduced under a P limitation due to a low C fixation rate, whereas the MC content was higher. Consequently, increases in the MC content per dry weight along with the production of the more toxic form, MC-LR, were observed under more P-limited conditions.

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. The toxin, a soluble peptide, is lethal to many kinds of aquatic organisms and damages zooplankton, fish (14), and the liver of higher animals (2, 22).

Many studies on the effects of environmental factors on microcystin production by cyanobacteria have been conducted with *Microcystis* (7, 13, 19, 20, 22–24), *Anabaena* (15, 16), *Oscillatoria* (17), and *Synechocystis* (11) species. The toxin of *M. aeruginosa* is at a maximum at light intensities between 40 and 50 microeinsteins m^{-2} s^{-1} (19, 23). The microcystin contents of *Anabaena* and *M. aeruginosa* are highest at 25°C (16) and between 20 and 24°C (21), respectively. Lower and higher temperatures decrease their amounts. In contrast, the effects of N and P on the toxin production by cyanobacteria are highly variable (13). Batch-cultured *M. aeruginosa* decrease in toxicity when N and inorganic C are removed from the medium (2). The concentration of microcystin in *Anabaena* increases with P (16). 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 (17). It was recently reported that the net microcystin production rate decreases as the specific cell division rate decreases in N-limited *M. aeruginosa* cultures (13). 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.

MATERIALS AND METHODS

M. aeruginosa UTEX 2388, obtained from the University of Texas at Austin, was investigated in a P-limited chemostat (working volume, 800 ml) at dilution rates ranging from 0.1 to 0.8/day at 28°C by using a medium modified from Smith and Wiedeman (18). The composition of the medium was 2,000 μM KNO_3, 6 μM Na_2HPO_4, 300 μM $MgSO_4 \cdot 7H_2O$, 100 μM $CaCl_2 \cdot 2H_2O$, 5,000 μM HEPES, 170 μM EDTA, 470 μM KOH (85%), 185 μM H_3BO_3, 100 μM $Na_2SiO_3 \cdot 9H_2O$, 17.9 μM $FeSO_4 \cdot 7H_2O$, and 1 ml of microelements composed of 8.82 g of $ZnSO_4 \cdot 7H_2O$, 1.44 g of $MnCl_2 \cdot 4H_2O$, 0.17 g of MoO_3, 1.57 g of $CuSO_4 \cdot 5H_2O$, and 0.4 g of $CoCl_2 \cdot 6H_2O$ in 1,000 ml of acidified water which included 1 ml of H_2SO_4 (98%) per 1,000 ml of distilled water. 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 fluorescence lamps at an average light intensity of 160 microeinsteins 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°C for further analysis.

The photosynthesis was measured by using the ^{14}C technique at an ambient light intensity of 160 microeinsteins m^{-2} s^{-1}. The radioactivity in the cells was measured by a liquid scintillation counter (Beckman LS6000). Chlorophyll a was extracted by using a chloroform-methanol mixture (2:1, vol/vol) and measured with a fluorometer (Turner 450) (25). The cell protein was determined by using bovine serum albumin as a standard (8). The cellular C was determined by using a total organic carbon analyzer (Shimadzu 5000A). The total N and P were determined after persulfate oxidation to nitrate (3) and orthophosphate (9), respectively. The nitrate was determined with a Szechrome NB reagent (26), and the orthophosphate was determined by the phosphomolybdate method (10).

The purification and analysis of the microcystins were carried out by the method developed by Harada et al. (6). First, the lyophilized cells were extracted three times with 50 ml of 5% (vol/vol) acetic acid for 30 min. The extract was centrifuged at 9,300 × g, and the supernatant was then applied to a C_{18} cartridge (Sep-Pak; Waters Association), eluted with methanol, and finally evaporated. The solution was analyzed on a high-performance liquid chromatograph (Shimadzu CLASS-LC10) fitted with a Nucleosil $5C_{18}$ column (Phase Separations) with a methanol–0.05 M phosphate buffer (pH 3.0) (57:43).

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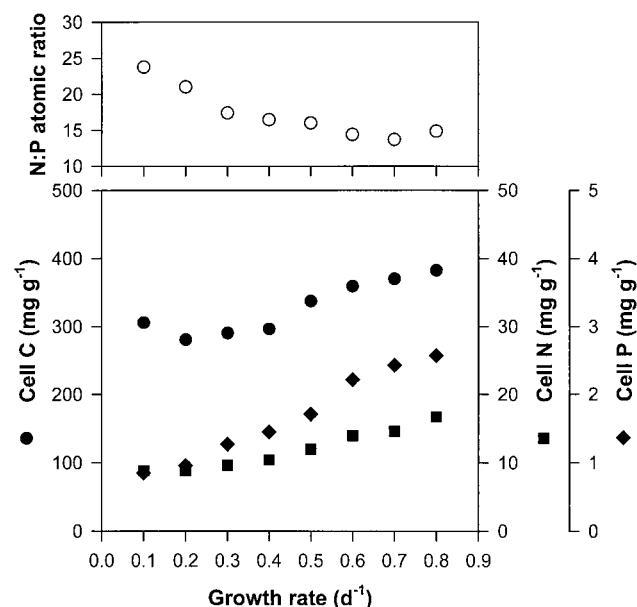
FIG. 1. N/P atomic ratio and cellular carbon, nitrogen, and phosphorus contents of *M. aeruginosa* at each μ in a P-limited chemostat.

RESULTS

P-limited growth. The changes in the cellular C, N, and P contents and the N/P atomic ratio of *M. aeruginosa* are shown in Fig. 1. On the basis of dry weight, cellular C, N, and P increased with a specific growth rate (μ); in particular, P increased about 3.0-fold with μ from 0.85 mg g^{-1} at μ of 0.1/day to 2.57 mg g^{-1} at μ of 0.8/day. The N/P atomic ratio of the steady-state cells was highest at μ of 0.1/day at 24 and gradually decreased with μ until 15 at μ of 0.8/day.

Microcystin production. The cell density and microcystin content of the steady-state cells of *M. aeruginosa* are shown in Table 1. The protein content slightly increased with μ and was highly correlated with μ ($Y = 38.0X + 134.0$; $r^2 = 0.746$). However, microcystins MC-LR and -RR were at a maximum at μ of 0.1/day at 339 μg g^{-1} and 774 μg g^{-1}, respectively, and then gradually decreased with μ, whereas no MC-YR was detected.

Figure 2 shows the relationship between the N/P atomic ratios and the microcystin contents. The cellular microcystin contents were highly correlated with the cellular N/P atomic ratios as follows: MC-LR ($Y = 25.8X - 262.0$; $r^2 = 0.968$), MC-RR ($Y = 31.9X - 5.4$; $r^2 = 0.955$), and MC-LR plus MC-RR ($Y = 57.7X - 267.3$; $r^2 = 0.979$).

The microcystin-producing rate, derived from multiplying the microcystin content by μ, increased linearly with μ ($Y = 456.8X + 81.6$; $r^2 = 0.991$), while the total microcystin content per unit of dry weight decreased with μ (Fig. 3). The maximum microcystin-producing rate was about 444 μg g^{-1} day^{-1} at μ of 0.8/day. The microcystin-producing rate of the cells increased about 4.0-fold when μ increased from 0.1 to 0.8/day, while the microcystin content decreased to 555 μg g^{-1} with μ, as a half of 1,118 μg g^{-1} at μ of 0.1/day. That is, the microcystin contents of the cells at a higher μ were low, while the microcystin-producing rate at a higher μ was higher due to the higher μ.

Photosynthetic activity. The C fixation rates of the steady-state cells of *M. aeruginosa* were investigated at an ambient light intensity of 160 microeinsteins m^{-2} s^{-1} (Fig. 4). The C fixation rates normalized into a chlorophyll *a* content increased linearly with μ. On a dry weight basis, the pattern of the C fixation rate versus μ was somewhat different from that on a chlorophyll *a* basis. The C fixation rates normalized into a dry weight increased with μ in two different patterns: (i) slight increase under μ of <0.5/day and (ii) dramatic increase under μ of >0.6/day.

Relationship between photosynthesis and microcystin production. The ratios of MC-LR to MC-RR and the C fixation rate to the microcystin-producing rate are plotted as a function of the N/P atomic ratio of the steady-state cells of *M. aeruginosa* in Fig. 5. When the N/P atomic ratio was high (severe P-limited condition), the MC-LR content was still lower than the MC-RR content (Table 1; Fig. 2), yet 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.

The microcystin-producing rates, on the basis of dry weight, increased dramatically with lower C fixation rates (2 to 4 mg of C g^{-1} h^{-1}); however, with higher C fixation rates (4 to 19 mg of C g^{-1} h^{-1}) the microcystin-producing rates were much reduced (Fig. 3 and 4). Consequently, there was a tendency of an increase in the ratio of the microcystin-producing rate to the C fixation rate in P-limited conditions (Fig. 5).

DISCUSSION

The cellular C, N, and P constituents of *M. aeruginosa* were changed as a function of μ in a P-limited chemostat. In particular, the cellular P concentration decreased dramatically with an increasing N/P atomic ratio, which meant further P limitation. As a result, the N/P atomic ratios of *M. aeruginosa* were within the range of 24 to 15, depending on μ from 0.1 to 0.8/day. In a previous study, Oh and Rhee (12) reported that the optimum N/P atomic ratio of *M. aeruginosa* was 11, which was determined as the ratio of minimum cell quotas of N to P.

TABLE 1. Cell density and microcystin content of *M. aeruginosa* grown in P-limited Smith and Wiedeman medium[a]

μ/day	Dry wt (mg/liter)	Protein content (mg/g [dry wt])	MC-LR		MC-RR	
			(μg/g [dry wt])	(mg/g of protein)	(μg/g [dry wt])	(mg/g of protein)
0.1	192 ± 4[b]	137 ± 8	339.2 ± 4.4	2.47 ± 0.03	773.5 ± 4.4	5.63 ± 0.03
0.2	163 ± 8	141 ± 4	297.7 ± 6.7	2.11 ± 0.05	666.5 ± 9.8	4.72 ± 0.07
0.3	79 ± 2	145 ± 5	181.1 ± 9.5	1.25 ± 0.07	522.9 ± 7.6	3.61 ± 0.05
0.4	62 ± 2	144 ± 2	174.1 ± 2.9	1.21 ± 0.02	495.4 ± 3.9	3.45 ± 0.03
0.5	48 ± 9	160 ± 8	153.2 ± 6.3	0.96 ± 0.04	478.9 ± 9.2	2.99 ± 0.06
0.6	20 ± 7	159 ± 4	113.5 ± 3.7	0.71 ± 0.02	481.3 ± 4.7	3.02 ± 0.03
0.7	14 ± 2	167 ± 4	104.2 ± 3.6	0.62 ± 0.02	463.6 ± 7.5	2.78 ± 0.04
0.8	11 ± 1	156 ± 5	87.9 ± 5.2	0.56 ± 0.03	467.0 ± 8.1	3.00 ± 0.05

[a] MC-YR was not detected.
[b] Each value in the table (except for those in the dry wt column) represents the mean of three measurements ± standard deviation.
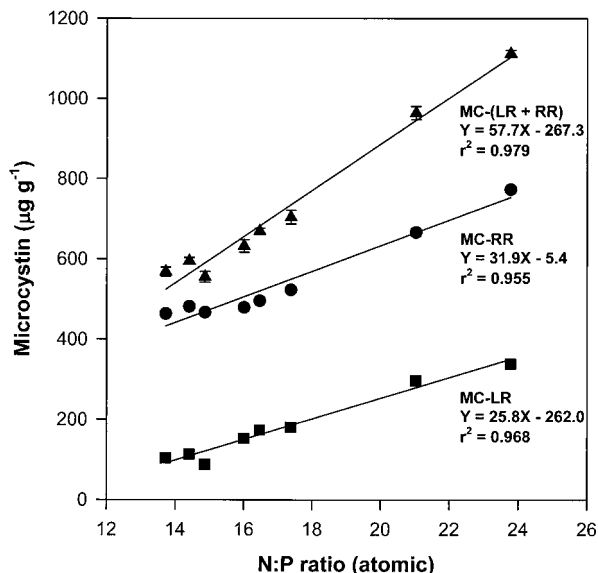
FIG. 2. Relationship between the N/P atomic ratios and the microcystin [MC-LR, MC-RR, and MC-(LR + RR)] contents of *M. aeruginosa*. The error bars indicate standard deviation ($n = 3$).

The microcystin content of the steady-state cells of *M. aeruginosa* decreased with μ, while the protein content slightly increased. That is, the production of microcystin may be uncoupled from general protein synthesis, as previously reported by Utkilen and Gjølme (19).

There have been several reports on the effect of P on microcystin production by cyanobacteria (17, 20, 23). Watanabe and Oishi (23) 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 (20) reported that phosphate-limited conditions have no effect on the toxin production of *M. aeruginosa*, whereas iron-limited conditions do. Recently, Sivonen (17) reported that the toxin production
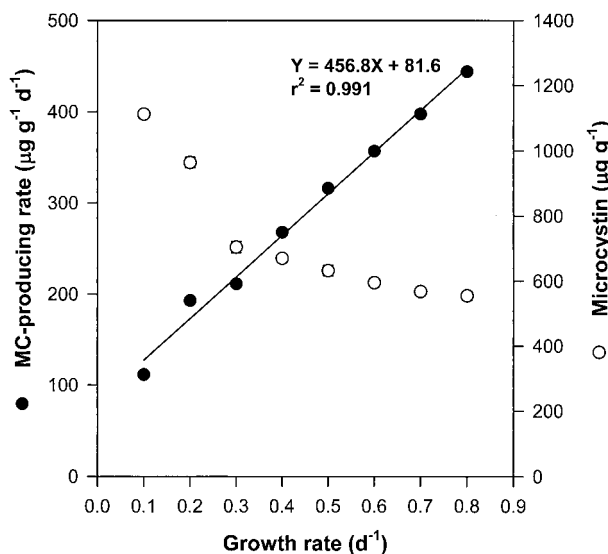
of *O. agardhii* depends on a low-level concentration of P (0.1 to 0.4 mg of P per liter) and higher concentrations have no additional effect. Accordingly, this indicates the possibility of an increase of toxicity in P-limited conditions. In the present experiment, the degree of the P limitation of the cells was directly analyzed, and the microcystin content of the cells at μ of 0.1/day increased to 1,113 μg g^{-1}, double that of 555 μg g^{-1} at μ of 0.8/day. In other words, more microcystins per unit of dry weight were produced in more P-limited conditions. From the above-mentioned results, it would seem that the controversies may arise from the following. (i) Complete cellular P limitation may not be induced by a supplied P concentration in a batch culture. (ii) There may be a threshold concentration of P which has an effect on the toxin production of cyanobacteria. (iii) There may be many differences among cyanobacterial species and strains.

There is a linear relationship between the microcystin-producing rate and μ ($Y = 456.8X + 81.6$; $r^2 = 0.991$). According to Watanabe et al. (22), the production rate of MC-RR in
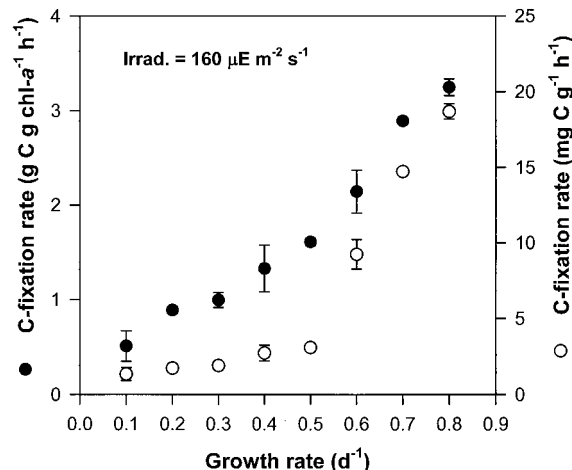

FIG. 4. C fixation rate on the basis of chlorophyll *a* (chl-*a*) and dry weight of *M. aeruginosa* at each μ in a P-limited chemostat. The ambient light intensity was 160 microeinsteins m^{-2} s^{-1}. The error bars indicate standard deviation ($n = 3$).

FIG. 3. Microcystin-producing rate and microcystin content of *M. aeruginosa* at each μ in a P-limited chemostat. The error bars indicate standard deviation ($n = 3$).
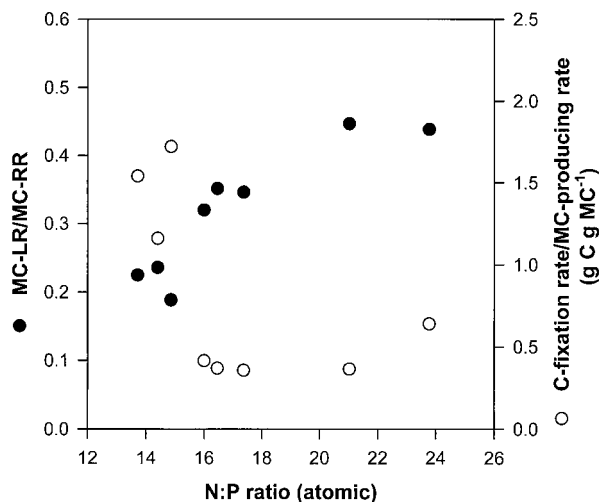

FIG. 5. Relationship between the N/P atomic ratios and the ratio of MC-LR to MC-RR or the C fixation rate to the microcystin-producing rate of *M. aeruginosa*.

Microcystis viridis was 175 μg g^{-1} day^{-1} and that of MC-YR in *M. aeruginosa* M228-12 was 1,130 μg g^{-1} day^{-1}. In the present experiment, the microcystin-producing rate of *M. aeruginosa* was highest at 444 μg g^{-1} day^{-1} at μ of 0.8/day. Therefore, it would seem that the microcystin-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.

At an ambient light intensity, there was a biphasic relationship between the C fixation rates based on their dry weights and growth rates. C fixed by photosynthesis is used not only for reproduction but also for cellular maintenance, and some is even excreted (4). The storage and utilization of fixed C may also vary depending on the degree of the P limitation. Therefore, it is not surprising that there was no straightforward relationship between growth and photosynthesis.

The ratio of MC-LR to MC-RR increased under severe P-limited conditions. Unfortunately, the toxicity of MC-LR is more than four times stronger than that of MC-RR judging from the data of the 50% lethal dose with mice (5, 7). According to Kotak et al. (7), MC-LR is one of the most potent microcystins, having a 50% lethal dose of 50 μg kg^{-1} by intraperitoneal injection in mice. The ratios of the microcystin-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 microcystin-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 microcystin-producing cyanobacterial bloom occurs frequently.

More P in the culture medium stimulates the growth and microcystin production of *M. aeruginosa* (Fig. 3). This finding can be extrapolated to *M. aeruginosa* growing under bloom conditions. That is, the reduction of P in eutrophic waters may lower the growth and microcystin-producing rate of *M. aeruginosa*, resulting in the reduction of the toxicity. Recently, a similar strategy was reported for *Anabaena* spp. (16).

The role of toxins in cyanobacteria is still not completely understood (16). It has been suggested that microcystins act as protective compounds against grazing by zooplankton (1). Therefore, it is postulated that, in the ecology of toxic cyanobacteria, *M. aeruginosa* under P-limited conditions has a more protective function by increasing toxicity against grazing by zooplankton. To confirm the above-mentioned opinion, further experiments are needed along with more information on the relationship between the microcystin content and type of *M. aeruginosa* and grazing by zooplankton.

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