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Effects of Environmental Factors on Toxicity of a Cyanobacterium (*Microcystis aeruginosa*) under Culture Conditions

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Effects of light intensity, temperature, and nutrients on the toxicity of *Microcystis aeruginosa* were investigated, using a toxic strain which kills mice. A marked change in toxicity was observed in the light intensity experiment, and slight changes were observed to be caused by temperature and phosphorus deficiency.

Accelerating eutrophication of waters results in blooms of planktonic algae, especially of blue-green algae (cyanobacteria). Such algae form dense surface films, and scums are often observed when wind blows the algal cells together. The death of domestic animals from ingesting algal cells has been often reported (11). Among the toxic blue-green algae, the water bloom of *Microcystis aeruginosa* is commonly observed in highly eutrophic lakes. Poisoning by *M. aeruginosa* has occurred in many countries of the world (5, 10, 11). The existence of toxic *M. aeruginosa* in lakes, ponds, and reservoirs in Japan was proven when we injected cell extracts of the alga intraperitoneally into mice (12). The animals' livers were engorged with blood, as reported by Konst et al. (9) and Heaney (7). The toxicity changed seasonally or from year to year. When we examined the toxicity of *M. aeruginosa* in Lake Suwa in Nagano Prefecture, the highest levels were observed from the middle of July to August; toxicity dropped in late August and September (14). Algae from the lake were toxic at a certain dose in 1977, but the same dose was not toxic in 1978 (14). There are two possible causes for this variation that can be considered: one is some environmental factor(s) that affects the toxicity; the other is a change in the algae population itself, either in the toxicity of an individual strain or in the composition of toxic and less-toxic strains within the population.

We have succeeded in isolating a highly toxic strain of *M. aeruginosa* (13). Using this strain, we carried out various experiments to establish the effects of environmental factors on toxin production.

The toxic strain M228 used in this study was isolated from an individual colony from the surface water of Lake Suwa in July 1978. By repeated washing with sterile medium and repeated passages, we obtained a unialgal but not axenic strain which grows in the form of single cells.

MA medium, reported by Ichimura (8), was used for isolation, preculture, and experiments. The composition of medium, per liter, was as follows: $Ca(NO_3) \cdot 4H_2O$, 50 mg; KNO_3, 100 mg; $NaNO_3$, 50 mg; Na_2SO_4, 40 mg; $MgCl_2 \cdot 6H_2O$, 50 mg; β-sodium glycerophosphate, 100 mg; P(IV) metals, 5 ml; H_3BO_3, 20 mg; Bicine, 500 mg (pH 8.6). A glass bottle containing 10 liters of MA medium was used as the culture vessel.

Culture was done at 25°C under continuous illumination of 30.1 microeinsteins (μE)/m^2 per s with no aeration or agitation (standard conditions). For phosphate deficiency experiments, the amount of β-sodium glycerophosphate was reduced to 1/10 or 1/20 of the original level. For nitrogen-deficient conditions, each of the three reagents of nitrate, calcium nitrate, sodium nitrate, and potassium nitrate, was reduced to 1/10 or 1/20 of the original medium concentration. Growth was measured by cell number using a Fuchs-Rosenthahl hemacytometer. The phycocyanin content of the cells was measured by the method of Hattori and Fujita (6). After the exponential phase had finished, the algal cells were harvested by centrifugation and freeze-dried. To determine the toxicity of the algal cells, freeze-dried cells were homogenized with water in a homogenizer (Polytron, Kinematica GmbH). The homogenates were allowed to stand overnight at 5°C, and after centrifugation, the supernatants were injected intraperitoneally to mice (ICR strain). The 50% lethal dose (LD_{50}) was calculated by probit analysis (3).

In a previous paper (13), we reported a highly toxic strain of *M. aeruginosa* (M228), which was used in the present study. As this strain has the highest toxicity after the exponential phase has finished, the cells were harvested at that point. Table 1 shows the change in the toxicity of *M. aeruginosa* under different conditions of light intensity. In each experiment we designated the standard conditions as 30.1 μE/m^2 per s at 25°C. Under standard conditions, the LD_{50} of the alga was estimated as 9.65 mg/kg. Similar values were estimated for cells grown under 75.3 μE/m^2 per s, whereas a lower toxicity was estimated for cells grown under 7.53 μE/m^2 per s.

Higher growth rates were observed at 32°C than at 25 and 18°C (Table 2). In the temperature experiment, the LD_{50} of the algal cells under the standard condition was 11.3 mg/kg. A little higher toxicity was estimated for the cells grown at 18°C, and the cells at 32°C showed a little lower toxicity. However, the change in toxicity under different temperature conditions was not so great as when light intensity was varied.

Figure 1 shows the growth curves of *M. aeruginosa* grown under nutrient-deficient conditions. As MA medium contains rather high amounts of nitrogen and phosphorus, these nutrients had to be reduced to 1/10 and 1/20 their original levels to provide nutrient-deficient conditions. In the phosphorus deficiency experiment, no difference was seen in the growth rates during the exponential phase, but the yields of the cells did change. In the nitrogen deficiency experiment, similar growth rates were observed for cells grown under standard and 1/10 nitrogen conditions during the first week, whereas the cells grown in 1/20 nitrogen medium showed retarded growth after day 4. At the final point in Fig. 1, the cells were harvested and tested for toxicity (Table 3). A little

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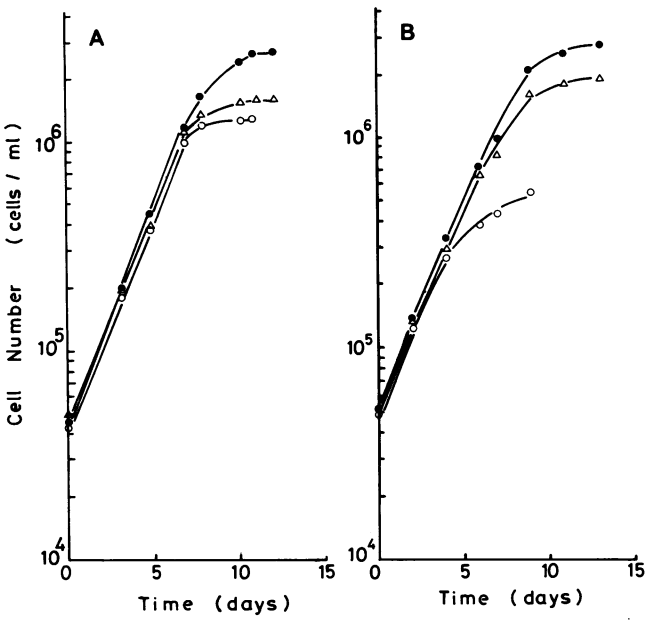

FIG. 1. Growth curves of a toxic strain of *M. aeruginosa* under (A) phosphorus- and (B) nitrogen-deficient conditions. Symbols: (A) (●) original concentration, (△) 1/10 phosphorus, (○) 1/20 phosphorus; (B) (●) original concentration, (△) 1/10 nitrogen, (○) 1/20 nitrogen.

lower toxicity was estimated for the phosphorus-deficient cells, but no difference was seen between the cells grown at 1/10 and 1/20 phosphorus conditions. In the nitrogen deficiency experiment, the LD_{50} of the cells under standard conditions was estimated as 10.4 mg/kg. Interestingly, the lowest toxicity was found with the cells grown in 1/10 nitrogen medium. The phycocyanin contents of the cells grown under standard, 1/10, and 1/20 conditions were 27.1, 6.9, and 11.6 μg/mg (dry weight), respectively.

In each experiment, we designated the standard conditions (25°C, 30.1 $\mu E/m^2$ per s, original nutrient concentration). Under these conditions, the LD_{50} ranged from 9.65 to 11.4 mg/kg and showed good stability.

Effect of culture age on toxicity. Gorham (4) stated that toxicity increases during exponential phase and then decreases. Eloff and Westhuizen (2) confirmed Gorham's statement in reference to the exponential phase, but showed that toxicity dropped more than 50% per day at the beginning of stationary phase. In a previous paper (13), we reported lowest toxicity levels in the middle of the exponential phase (LD_{50}, 68.1 mg/kg), LD_{50} of 11.7 mg/kg at the beginning of stationary phase, and LD_{50} of 18.4 mg/kg 8 days after. Thus, because of the stability of LD_{50} under the standard conditions of this study, we suppose that the toxicity decreased gradually during the stationary phase.

TABLE 1. *M. aeruginosa* toxicity and growth under different conditions of light intensity

Light intensity ($\mu E/m^2$ per s)	LD_{50} (mg/kg)	μ/day
7.53	36.9 (29.7–41.4)[a]	0.25
30.1	9.65 (8.36–10.6)	0.52
75.3	9.97 (7.99–11.5)	0.90

[a] 95% confidence limit.

TABLE 2. *M. aeruginosa* toxicity and growth at different temperatures

Temp (°C)	LD_{50} (mg/kg)	μ/day
18	10.9 (9.05–12.8)[a]	0.37
25	11.3 (9.65–13.1)	0.44
32	15.3 (13.3–57.7)	0.59

[a] 95% confidence limits.

This work was done to determine the influences of environmental factors on the toxicity of *M. aeruginosa*. According to Gorham (4), the difference in toxicity was not significant between cells grown at 200 ft-c (ca. 2,150 lx) and at 1,600 ft-c (ca. 17,220 lx). In our experiment, algal cells grown under 30.1 and 75.3 $\mu E/m^2$ per s showed similar toxicity, whereas the toxicity of cells grown under 7.53 $\mu E/m^2$ per s was reduced to the lowest level of the present study. Gorham (4) also demonstrated the highest toxicity of cells at 25°C, the lowest at 20°C (about one-fifth of the value at 25°C), and reduction of one-half at 30°C, though his toxicity was expressed as mouse units. In the present experiment, we observed a slight increase in toxicity at low temperature and a slight decrease at high temperature.

In the nitrogen-deficient conditions, the cells grown at 1/10 nitrogen concentration were less toxic than those grown at 1/20 nitrogen. The phycocyanin content of cells grown at 1/20 nitrogen, however, was higher than that at 1/10 nitrogen. Allen and Smith (1) reported that nitrogen-deficient conditions led to the decrease of phycocyanin content in blue-green algae. Although the phycocyanin content of the cells grown in 1/10 nitrogen medium was lower than that in 1/20 nitrogen medium, the growth curves of the cells showed that the nitrogen limitation was more severe in the 1/20 medium than in the 1/10 one. At the final point of each growth curve, the dry weights of algal cells per liter were 41.6, 32.6, and 17.5 mg for the standard, 1/10, and 1/20 nitrogen conditions. Since, for this experiment, the nitrogen limitation was brought about by reducing the amount of nitrate per volume of medium, the dry weights of algal cells at the final points were divided by the LD_{50} values. This resulted in values of 4.00, 1.26, and 1.10 kg/liter for the

TABLE 3. *M. aeruginosa* toxicity under nutrient-deficient conditions

Nutrient	Concn	LD_{50} (mg/kg)
Phosphorus	Original	11.4 (9.27–13.3)[a]
	1/10	14.4 (12.4–16.5)
	1/20	14.4 (12.4–16.7)
Nitrogen	Original	10.4 (6.77–12.8)
	1/10	25.5 (22.0–30.9)
	1/20	15.8 (13.5–18.0)

[a] 95% confidence limits.

standard, 1/10, and 1/20 conditions, respectively, indicating that the toxin content per liter of medium was similar in both the 1/10 and 1/20 nitrogen conditions. We think further experiment will be needed to explain this similarity.

Throughout the present study, a marked change in toxicity was observed in the light intensity experiments. Production of the toxin was suppressed under low light intensity. However, similar LD_{50} values were estimated for cells grown under 30.1 and 75.3 $\mu E/m^2$ per s. This means that light intensity is the primary important factor for the production of the toxin; however, it seems that high toxin production can occur at relatively low light intensity.

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