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Increased Microcystin Production of *Microcystis aeruginosa* by Indirect Exposure of Nontoxic Cyanobacteria: Potential Role in the Development of *Microcystis* Bloom

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Phytoplankton succession is strongly affected by abiotic and biotic parameters, including availability and competition for resources, selective grazing, and interactions within algal group (Reynolds 1984). The latter includes a kind of allelopathic interaction among algal communities in which chemicals produced and excreted by one algal species affect the proliferation of others and thereby alter the population composition and dynamics (Keating 1977; Sukenik et al. 2002). In eutrophic freshwaters, *Microcystis aeruginosa* gains advantage over other cyanobacterial species (*Anabaena*, *Planktothrix*) and then forms blooms during dry summer seasons. Previous studies showed that, once a population of toxic *Microcystis* forms a bloom, it could inhibit growth of other algae (Singh et al. 2001).

Microcystins are hepatotoxins produced by bloom-forming cyanobacteria, primarily *Microcystis* species. These compounds include defense or deterrence against grazers, symbiotic relationships, metal acquisition and storage, and reserve pools of metabolites (Codd 1995). An increasing number of studies have been conducted in order to determine the ecological interactions of cyanobacteria and aquatic organisms. Recently, increased microcystin production was observed in several *M. aeruginosa* strains as an "induced defense" mediated by chemicals from zooplankton or fish (Jang et al. 2003, 2004). Mohamed (2002) also revealed the allelopathic activity of the green alga *Spirogyra* simulating the growth and microcystin production by toxic *Oscillatoria*. However, there is little information on the microcystin production mechanism of *Microcystis* associated with interactions of other nontoxic cyanobacteria.

The purpose of this study was to evaluate changes in microcystin production by strain of toxic *M. aeruginosa* in response to indirect exposure to two nontoxic cyanobacteria (*Anabaena flos-aquae* and *Planktothrix agardhii*), which are frequently observed before *Microcystis* blooms in eutrophic freshwaters. We test a hypothesis that toxin production of *M. aeruginosa* could be mediated by chemicals from other cyanobacteria.

MATERIALS AND METHODS

Three cyanobacterial strains, *M. aeruginosa* (strain 88), *A. flos-aquae* (strain 74),

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and *P. agardhii* (strain 204, from the Microbial Culture Collection, National Institute for Environmental Studies, Japan), were used for this study. Although strain 88 has been reported as "toxic", strains 74 and 204 are "nontoxic" (Kasai et al. 2004). These strains are axenic and monoalgal. Each strain was axenically grown in batch culture in CT medium at 27°C with a light–dark regime of 16 h:8 h (irradiance, 120 $\mu mol/m^2/s$). The composition of CT medium was described in Jang et al. (2004).

For determination of the toxic *Microcystis* response to indirect exposure of nontoxic cyanobacteria, *M. aeruginosa* were separately inoculated in flasks with 200 mL of CT medium (control) or CT medium containing 100 mL of *A. flos-aquae* or *P. agardhii* culture media filtrate (*A. flos-aquae* culture media filtrate or *P. agardhii* culture media filtrate treatments; 50% of the total volume of culture media was ACMF or PCMF and the reminder was standard CT media). Triplicates of each treatment (3 × 2 species × 5 days; i.e. day 2, 4, 6, 8, and 10) as well as the control (3 × 6 days; i.e. day 0, 2, 4, 6, 8, and 10) were used (a total of 48 flasks). The algal cultures within the flasks were shaken by hand four times a day during the 10-day experiment.

M. aeruginosa biomass, nutrient concentrations (NO_3-N, NO_2-N, NH_4-N, and PO_4-P), intracellular microcystin, and extracellular microcystin were analyzed every 2 days until day 10, when the experiment was terminated. Algal biomass was monitored by measuring freeze-dry weight. Water samples for nutrient analysis were passed through GF/F and then measured according to APHA methods (1995). Intracellular microcystin was extracted from *M. aeruginosa* cells by filtration through glass-fiber filters (GF/C, Whatman, UK). Cells were harvested by centrifugation at 12000*g* at 4°C, freeze-dried, weighed on a balance (PB303-S Delta Range, Mettler Toledo, USA), and then stored at −70°C until analysis for microcystin. Extracellular microcystin was extracted from filtered water (through GF/C) through a 1.431-g Oasis® HLB 1-cc extraction cartridge (Waters, USA) and then stored in a refrigerator at 4°C until microcystin analysis. Details of microcystin analysis are based on Oh et al. (2000), and Jang et al. (2003, 2004).

Intracellular microcystin was extracted from freeze-dried *M. aeruginosa* cells twice with 30 mL of 5% (*v/v*) acetic acid for 16 h while shaking at 140 rpm. The extract was centrifuged at 12,000*g*, and then supernatant was applied to an extraction cartridge (Waters, USA). Those for intracellular microcystin and extracellular microcystin were eluted with methanol, and then evaporated. Finally, the solutions were analyzed on a HPLC (Waters 2690, Waters 996 Photodiode Array Detector, USA). The separation was performed on a Capcellpak C_{18} (4.6 mm × 150 mm, 5.0, Shiseido, Tokyo, Japan) reverse-phase column and the mobile phase was methanol-0.05 mol/L phosphate buffer (58:42, pH 3.0). The microcystin was identified by their UV spectra and retention times, and by spiking the sample with purified standards of microcystin-LR (Wako, USA) and microcystin-RR (Wako, USA). In this study, the sum of microcystin-LR and microcystin-RR was expressed as 'microcystin'. The detection limit for intracellular microcystin and extracellular microcystin were 0.1 μg/g dry cell and 0.1 μg/mL, respectively. Each analysis was performed in duplicate. Intracellular microcystin and extracellular microcystin were

expressed as μg/g dry cell and μg/mL of water samples, respectively.

Differences in *M. aeruginosa* biomass, intracellular microcystin and extracellular microcystin between the control and treatments were assessed using repeated-measured ANOVA and Tukey test. ANCOVA was performed to estimate differences in regression lines between control and treatments. The data on day 0 are excluded from the analysis (SPSS Release 12.0; SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Overall, the *M. aeruginosa* biomass remained or increased over the course of the experiment (Fig. 1A). There were no significant differences in the biomass between the control and the treatments ($F_{2,42}$ = 1.321, P = 0.278). Dry weights of the treatments sharply increased between days 2 and 4 and then remained approximately stable or decreased over the course of the experiment. No significant differences in DIN (Dissolved Inorganic Nitrogen; NO_3-N + NO_2-N + NH_4-N) and DIP (Dissolved Inorganic Phophorus; PO_4-P) concentrations were observed between the control and the treatments. Changes in nutrient availability, which would lead to an indirect exposure effect, were not observed until the end of the experiment, showing 31 mg/L on the initial day to 28 mg/L on the final day of DIN and 1.4 to 2.0 mg/L of DIP.

Significant increase in the intracellular microcystin was observed for the strain by the exposure of two species of cyanobacteria culture medium filtrates ($F_{2,42}$ = 6.035, P = 0.005; P = 0.018 for ACMF and P = 0.008 for PCMF; Fig. 1B). Amounts of intracellular microcystin peaked on day 4 in both the control and the treated samples (the mean values of the control, ACMF, and PCMF are 7139, 8096, and 7505 μg/g dry cell, respectively). Subsequently, they gradually decreased until the end of experiment. The higher intracellular microcystin of the treatments compared to controls could be associated with chemical signals from *Anabaena* culture media filtrates (ACMF) or *Planktothrix* culture media filtrates (PCMF). The microbial activity of chemicals declined within 24 h at 37°C, but it could be maintained longer at the lower temperature (Mitchell and Carvalho 2002). The temporal patterns of released extracellular microcystin were almost similar to those of intracellular microcystin, indicating significant differences between the control and the treatments ($F_{2,42}$ = 5.383, P = 0.008; Fig. 1C). On the day of the peak level, the total extracellular microcystin contents containing ACMF or PCMF were significantly greater than the control (mean value of 141 μg/g dry cell in control; 184 μg/g dry cell in ACMF; 193 μg/g dry cell in PCMF). Bioassay by Singh et al. (2001) showed that addition of 50 μg/mL of purified microcystin inhibited growth of cyanobacteria. Based on our data, the growth inhibition level (50 μg/mL) was detected in the media from day 2, then the 140-160% higher extracellular microcystin were released between days 4 and 8 when exposed to the cyanobacteria culture media filtrate (CCMF) (Fig. 1C).

Intracellular microcystin and extracellular microcystin showed a linear regression pattern, reflecting extracellular microcystin production by *Microcystis* that increased

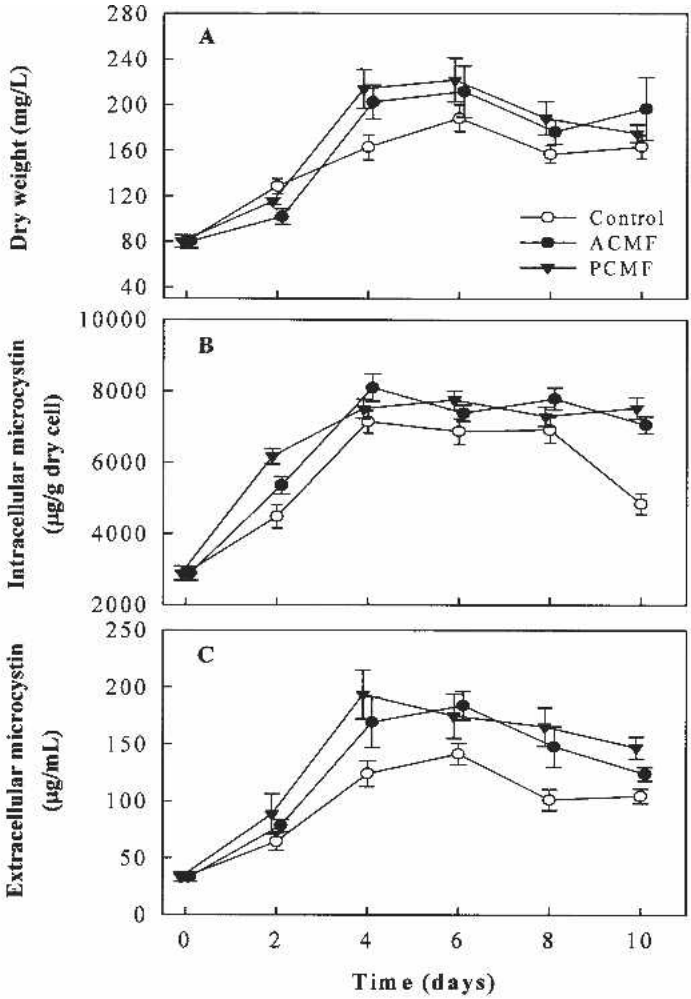

Figure 1. Changes in dry weight (A), amounts of intracellular microcystin (B), and extracellular microcystin (C) of *M. aeruginosa* when exposed by nontoxic *A. flos-aquae* culture media filtrate (ACMF) or *P. agardhii* culture media filtrate (PCMF) for 10 days. Data are man and SE (n=3)

with increasing intracellular microcystin by exposure of ACMF or PCMF (Fig. 2).

The y intercept of the control and ACMF was not significantly different (ANCOVA, $P = 0.111$), but both slopes were significantly different ($P = 0.076$). The slope of PCMF was significantly higher than that of the control (ANCOVA, $P = 0.008$), and

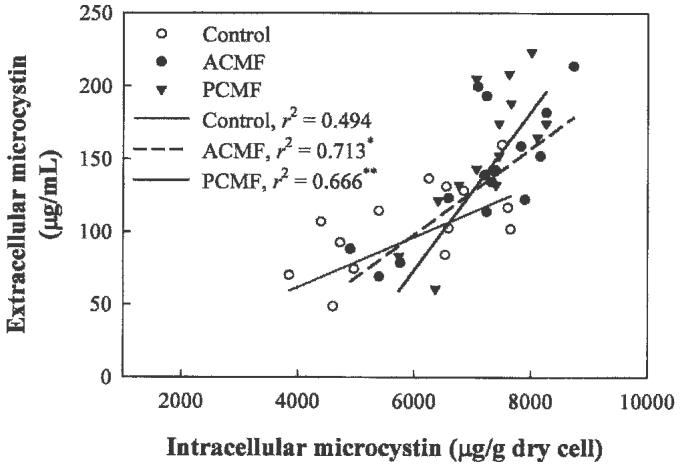

Figure 2. Relationships between intracellular microcystin and extracellular microcystin of *M. aeruginosa* by indirect exposure of *A. flos-aquae* culture media filtrate (ACMF) and *P. agardhii* culture media filtrate (PCMF) over the course of the experiment. Significance differences in slopes between treatments and control are indicated by $^*P < 0.1$ and $^{**}P < 0.01$.

the intercept of PCMF was also lower than that of the control ($P = 0.015$). Therefore, the extracellular microcystin of PCMF and ACMF were significantly higher than that of the control within a high range of intracellular microcystin (ca. 6000 μg/g dry cell).

Until now, uncertainties had remained on the release mechanisms of extracellular microcystin, except for cell lysis. From our study, we postulate that increased extracellular microcystin from toxic *Microcystis* were released mediated with unknown chemicals by the allelopathic activity of nontoxic cyanobacteria. Although variations of extracellular microcystin amounts may be of minor importance from the toxicological point of view (Dittmann and Börner 2005) owing to their very low amounts (below ~10% of total microcystin), their ecological roles have to be considered for biological interactions in eutrophic freshwaters.

Our results conclusively reflected that potentially toxic cyanobacteria may increase production of intracellular and extracellular microcystin in response to chemicals from competitive nontoxic cyanobacteria. This is the first suggestion that allopathic interactions occur within cyanobacteria species. Although complex environmental parameters are involved in the toxic *Microcystis* bloom formation, once a toxic *Microcystis* form blooms, it may depress the growth of *Anabaena* or *Planktothrix* by its physiological characteristics; proliferation of the toxic *Microcystis* bloom would then be observed in eutrophic freshwaters.

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