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Microcystin Production by *Microcystis aeruginosa* Exposed to Phytoplanktivorous and Omnivorous Fish at Different Kairomone Concentrations

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Abstract We investigated microcystin (MC) production by three *Microcystis aeruginosa* strains in response to different kairomone concentrations (indirect exposure: 0%, 10%, 25%, and 50%) of *Hypophthalmichthys molitrix* and *Carassius gibelio langsdorfi*. The MC production was significantly different among control and three kairomone treatment levels. MC production was higher with increasing concentration of kairomones and the peak intracellular and extracellular MC contents showed clear dose-dependent response ($p < 0.05$). This study provides the first indirect evidence of increased MC production of *M. aeruginosa* in response to increasing kairomone concentrations released by phytoplanktivorous and omnivorous fish.

Keywords Microcystin · Kairomone · *Hypophthalmichthys molitrix* · *Carassius gibelio langsdorfi*

Aquatic organisms are exposed to a wide range of water-soluble chemicals such as kairomones or infochemicals. The chemicals are produced by predators that affect characteristics of prey species (Dodson et al. 1994), then prey organism defend themselves against predators through alteration of their morphology, behavior, physiology and life history. This inducible defence can be important ecological factors, with both direct and indirect effects at the community level. One way to assess the risk of predation is to trace chemical cues (kairomones, for terminology see Dicke and Sabelis 1988) released by active predators. Such chemical cues may convey information about species identity and feeding habits of the predator. Recent observations indicate that induced chemical defence widespread and occur in plants and animals from freshwater habitat (Tollrian and Harvell 1999). However, experimental studies on chemical-medicated interactions in phytoplankton and fish are scarce.

Recently, Jang et al. (2004) found that potentially toxic cyanobacteria increase their toxin production when directly exposed to fish, especially phytoplanktivorous species, even though fish appeared not to feed vigorously on toxic *Microcystis*, as a fish-induced defence mediated by physical contact associated with feeding or by chemical cues. In this study, using fish indirect exposure experiments, we investigated whether toxin production by three strains of *Microcystis aeruginosa* depends on kairomone concentration of two different feeding types of fish (planktivorous *Hypophthalmichthys molitrix* and omnivorous *Carassius gibelio langsdorfi*), and our aim was to test the hypothesis that MC production by *M. aeruginosa* increases in response to increasing kairomones released by fish.

Materials and Methods

Three strains of *M. aeruginosa* (Strains 89, 103 and 107 from the Microbial Culture Collection, National Institute for Environmental Studies, Japan) were used. All three strains are axenic and monoclonal (Kasai et al. 2004). Each strain was axenically grown in batch culture in CT medium

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(adjusted pH 8.2) at 27°C in an incubator with a light-dark (LD) regime of 16:8 h (irradiance, 120 μmol m^2/s). The composition of CT medium was described in Jang et al. (2007). *H. molitrix* and *C. gibelio langsdorfi* were obtained from a fish farm in Saitama, Japan. The two species were held separately in a 50 L aerated recirculation glass tanks containing dechlorinated water 1 week before the experiment. The water quality characteristics were described in Jang et al. (2004). During rearing prior to the experiment, *H. molitrix* were fed with *Scenedesmus* and *C. gibelio langsdorfi* with commercial pellet diet (Crumble 4C, Nihon-Haigou-Shiryou Co., Ltd., Yokohama, Japan), once daily between 09:00 and 09:30 h, respectively. Fish used in this experiment were starved for 24 h before starting the experiments to ensure complete gut evacuation.

Microcystin production in the three strains of *M. aeruginosa* was compared between fish culture media filtrates (FCMF) and FCMF-free controls. Twenty-four individuals (8 fish were used for each series of experiment) of *H. molitrix* (mean ± SE.; total length, 98.9 ± 1.5 mm; weight, 7.1 ± 0.3 g) and 24 individuals of *C. gibelio langsdofi* (mean ± SE.; total length, 87.9 ± 1.1 mm; weight, 9.1 ± 0.4 g) were used for this experiment. To obtain fish culture media filtrates containing dissolved chemicals released from fish, eight individuals of *H. molitrix* or *C. gibelio langsdofi* were incubated for 3 days, in 12 L of dechlorinated water. After removal of the fish, the water was passed through a 0.45 μm GF/F filtration and then a nucleopore filter (0.2 μm pore size; Whatman) in a sterilized room to remove algal cells, bacteria, and other particulates. To determine the cyanobacterial response to indirect fish exposure, the three strains of *M. aeruginosa* were separately cultures in flasks with 300 mL of CT medium and three levels of *H. molitrix* filtrate (HCMF1, HCMF2, and HCMF3: 10%, 25%, and 50% of the total volume of culture media was *H. molitrix* filtrate, respectively, and the reminder was standard CT media) and *C. gibelio langsdofi* filtrate (CCMF1, CCMF2, and CCMF3: 10%, 25%, and 50%, respectively). Triplicates of each treatment (3 × 3 levels of FCMF × 2 fish species × 6 days) as well as a control (3 × 7 days including day 0) containing no FCMF (a total of 129 flasks) were used for each strain. The algal cultures within the flasks were shaken four times daily. Three flasks from the control and each treatment (a total of 21 flasks) were chosen randomly each day for analysis.

Microcystis aeruginosa biomass, intracellular and extracellular MC were analyzed daily until day 6. Nutrient (NO_3-N, NO_2-N, NH_4-N, and PO_4-P) concentrations were analyzed on days 0 and 6 (n = 48). The water samples for NO_3-N, NO_2-N, NH_4-N, and PO_4-P were passed through a GF/F filter and then measured according to standard methods (APHA et al. 1995). *M. aeruginosa* biomass was monitored by measuring freeze-dried weight. For analysis of intracellular MC, cells were harvested by centrifugation at 12,000*g* at 4°C, freeze-dried, weighted on a balance (PB303-S Delta Range, Mettler, Toledo, USA), and then stored at −70°C until analysis for MC. To obtain extracellular MC, the flask water was passed through a GF/C filtration, using a 1.431 g Oasis HLB 1 cc extraction cartridge (Waters, Milford, USA) and then cartridge samples were stored at 4°C until MC analysis. Preliminary analyzes of FCMF from the same stocks as those used in the experiment showed no MC for 6 days before the experimental exposure to cyanobacteria. The DIN and DIP concentrations of filtered fish-cultured waters were 3.9–5.3 and 0.1–0.3 mg/L (n = 3), respectively.

Intracellular MC was extracted twice from freeze-dried *Microcystis* cells with 30 mL of 5% (*v/v*) acetic acid for 16 h while shaking at 140 revolutions per minute. The extract was centrifuged at 12,000*g*, and the supernatant was applied to a 1.431 g Oasis HLB 1 cc extraction cartridge (Oasis, Waters). The supernatants for intracellular and extracellular MC were eluted with methanol and then evaporated. Finally, the solutions were analyzed by high-performance liquid chromatography (Waters 2690, Waters 996 Photodiode Array Detector). Separation was performed on a Capcellpak C_{18} (4.6 × 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 MCs were identified by their ultraviolet spectra and retention times and by supplementing the sample with purified standards of MC-LR and MC-RR (Wako). In this study, the sum of MC-LR and MC-RR was expressed as 'MC'. The detection limits for intracellular and extracellular MC were 0.1 μg/g dry weight and 0.1 μg/mL, respectively. Each analysis was performed in duplicate. The intracellular MC concentration was expressed as μg/g dry cell and extracellular MC was expressed as μg/mL of water samples.

Differences in *M. aeruginosa* biomass, intracellular and extracellular MC among the control and the fish treatments over time were assessed by using a repeated-measurement analysis of variance (RM-ANOVA), and post hoc comparison were performed using Tukey multiple tests. Data gathered on day 0 were excluded from the analysis.

Results and Discussion

The exposure to FCMF stimulated the growth of all three *M. aeruginosa* strains (Figs. 1a–c, 2a–c), and the change in biomass differed significantly among the three FCMF concentrations (RM-ANOVA and post hoc Tukey test, $p = 0.001$). The patterns of nutrient concentration showed a decrease in DIN (from 62–65 mg/L on day 0 to 35–55 mg/L on day 6) and an increase in DIP (from 1.0–1.5 mg/L on day

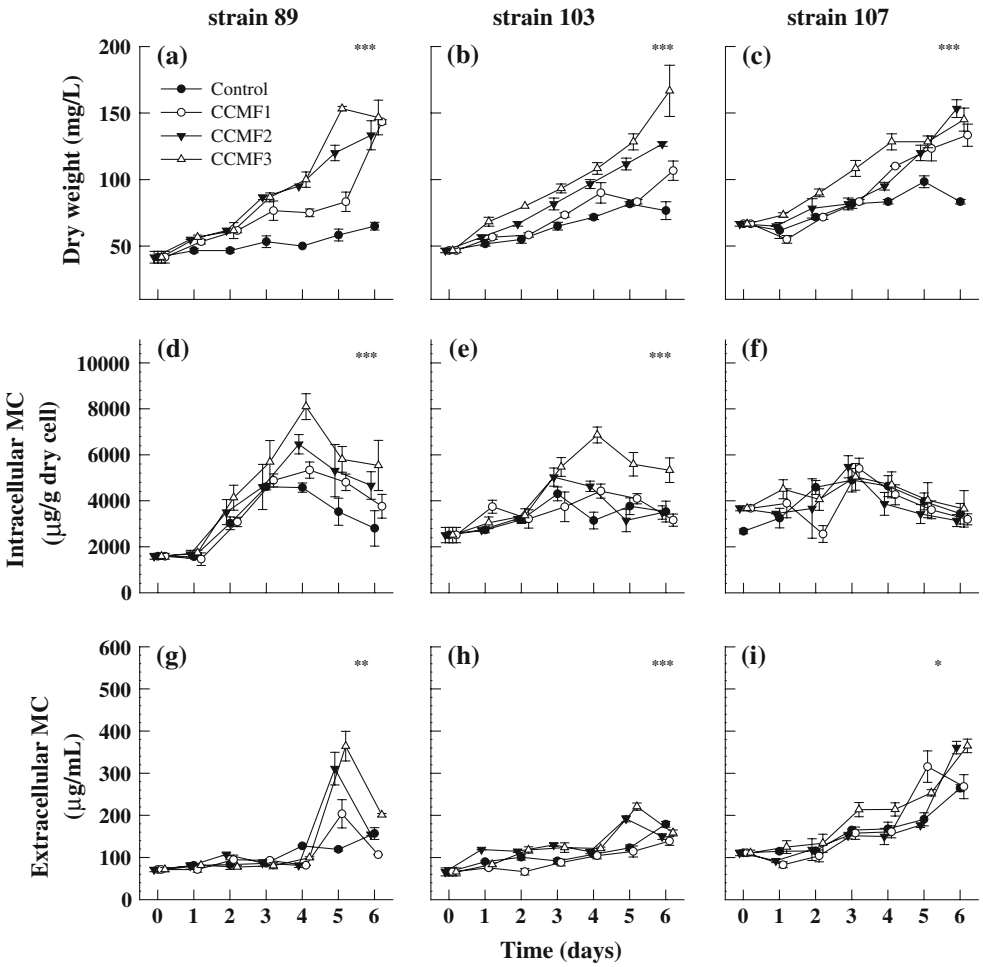

Fig. 1 Changes in dry weight, intracellular MC and extracellular MC when three *M. aeruginosa* were exposed to three concentrations (10%, 25%, 50%) of *C. gibelio langsdofi* culture media filtrate (indirect exposure) or the control. Data are means ± SE (n = 3). Significant differences between controls and treatments on the basis of RM-ANOVA test are indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

0 to 1.1–4.9 mg/L on day 6) over the course of the FCMF experiment. Intracellular MC levels were significantly different among the control and three levels of FCMF treatments (Figs. 1d–f, 2d–f). Over the course of the experiment, the intracellular MC levels of all strains significantly increased after exposure to FCMF ($p < 0.05$), except in the case of strain 107 with *Carassius* culture media filtrate. The MC production was higher with increasing concentration of FCMF. The MC levels of all three strains peaked at days 3 and 4 in all FCMF treatments. On the peak day, the intracellular MC levels showed clear dose-dependent response ($p < 0.05$), except in the case of strain 107 in the treatment of CCMF (Figs. 1d–f, 2d–f). Extracellular MC concentrations were much lower than intracellular ones, but both showed similar temporal patterns over the course of the experiment. For all three strains the extracellular MC levels were significantly different among the control and three treatments, and the levels increased after the exposure to FCMF (Figs. 1g–i, 2g–i). The extracellular MC levels of all strains peaked at day 5, except in the only two cases of strain 107 with CCMF2 and CCMF3. On the peak day, the extracellular MC levels showed clear dose-dependent response ($p < 0.05$), except in the case of strain 107 in the treatment of CCMF (Figs. 1g–i, 2g–i).

This study provides the first indirect evidence of increased MC production of *M. aeruginosa* in response to increasing kairomone concentrations released by fish and this response was stronger in phytoplanktivorous *H. molitrix* than omnivorous *C. gibelio langsdorfi*. This concentration dependent inducible defences provide good support for our earlier study (Jang et al. 2004) in which we found several strains of *M. aeruginosa* increased the level of MC when directly exposed to fish.

Under normal culture conditions, microcystin content was closely related to the cell growth pattern, showing toxin production of *M. aeruginosa* generally peaked during the exponential growth stage (Lee et al. 2000). However, our results showed that the intracellular and extracellular MC productions in *M. aeruginosa* strains tended to peak on the 3–5 days in response to indirect exposure of fish, and then decreased afterwards soon, although the amount of toxins produced differed among the three *M. aeruginosa* strains. This increase and subsequent decline in toxin production during the 6-day experiments may be due to the

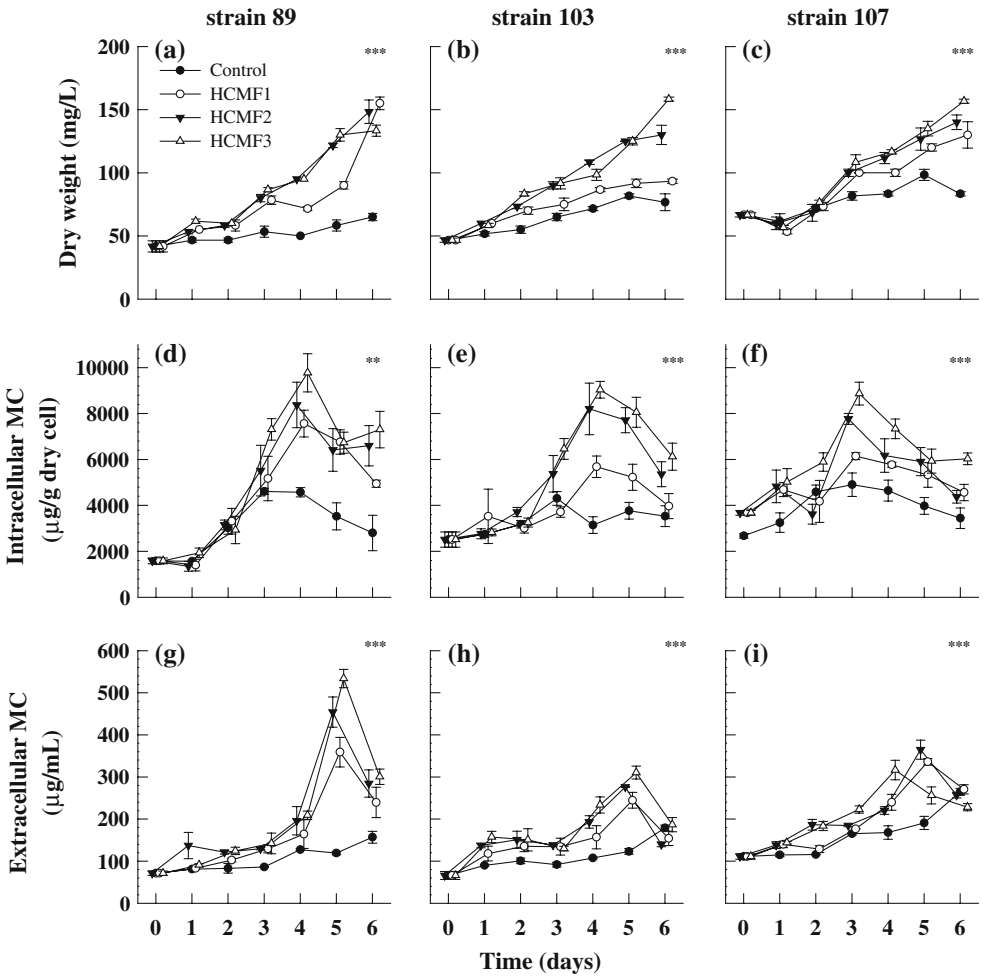

Fig. 2 Changes in dry weight, intracellular MC and extracellular MC when three *M. aeruginosa* were exposed to three concentrations (10%, 25%, 50%) of *H. molitrix* culture media filtrate (indirect exposure) or the control. Data are means ± SE (n = 3). Significant differences between controls and treatments on the basis of RM-ANOVA test are indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

degradation of chemical signals from fish. The chemicals (kairomones) from fish or fish culture media filtrates that affect morphological changes in zooplankton (*Daphnia*) are known to degrade after a few days (Loose et al. 1993).

This finding suggests that cyanobacteria release MC into the extracellular environment as an induced defence against fish, and that this release is triggered by kairomones produced by the fish. From a toxicological point of view (Dittmann and Börner 2005), variations in the levels of extracellular MC may be of minor importance owing to their very small amounts (<∼10% of total MC), but the MC can severely affect other organisms when released into the surrounding waters. Therefore, the ecological roles of extracellular MC must be considered in studies of the biological interactions of toxic cyanobacteria and other food-web components in eutrophic freshwaters.

Our results showed species-specific effect and they did reveal marked differences in both intracellular MC production and extracellular MC production by varying kairomone concentrations within the same fish species. This dose-dependent inducible defence revealed in this fish indirect-exposure experiment might provide valuable information in the context of toxic cyanobacterial ecology.

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