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Microcystin production by *Microcystis aeruginosa* exposed to different stages of herbivorous zooplankton ☆

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Abstract

Microcystin (MC) production by four monoclonal *Microcystis aeruginosa* strains was evaluated in response to infochemicals (indirect exposure) released from different stages of herbivorous zooplankton (neonate/juvenile and adult *Daphnia magna* and *Moina macrocopa*). The intracellular MC and extracellular MC concentrations were significantly different among the control and treatments with zooplankton culture media filtrates ($p<0.05$), and in most cases MC production was significantly higher ($p<0.05$) in strains exposed to infochemicals released from adult zooplankton rather than those of neonate/juvenile zooplankton in four strains of *M. aeruginosa*. Compared to intracellular MC (385.0–5598.6 $\mu g\,g^{-1}$ DW), very low concentrations of extracellular MC (9.9–737.6 $\mu g\,ml^{-1}$) were released, but both showed similar temporal patterns over the course of the experiment. This result might be attributed to the fact that adult zooplankton produced more infochemical signals than equal numbers of smaller juveniles and neonates. It is the first study to provide evidence that MC production might be impacted by infochemicals released from different stages of zooplankton, mediated with physiological characteristics, body size, and feeding habits.
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Keywords: *Microcystis aeruginosa*; Induced defense; Different stages of zooplankton; Infochemicals; Intracellular microcystin; Extracellular microcystin

1. Introduction

Inducible defenses can be important ecological factors, with both direct and indirect effect at the community level. Herbivory by zooplankton is the strongest selective pressure acting on phytoplankton, and there are several examples of defenses (e.g., production of chemical deterrents, morphological changes including colony formation) induced by herbivores' infochemicals in phytoplankton (van Alstyne, 1988; Hessen and van Donk, 1993; Lürling and van Donk, 1997; van Donk et al., 1999; Wolfe

☆ *Ethical statement*: Submission of this manuscript implies that the work described has not been published before and that it is not under consideration for publication elsewhere. The corresponding author, Dr. Min-Ho Jang, ensures that its publication has been approved by co-author Dr. Noriko Takamura and by the responsible authorities in the laboratories where this work was carried out. Therefore, this article will not be published elsewhere in the same form in either the same or other language. We are responsible for at least the part describing our contribution and have seen the entire final text before submission. We have drawn attention to chemical or biological hazards that may be involved in materials and methods used in this experiment.

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et al., 1997; Jang et al., 2003; Tang, 2003). The induced antipredator morphological defenses is dependent on consumer density and is triggered by infochemical cues (Lampert et al., 1994; van Donk et al., 1999), although the identity of these chemicals is still under debate.

Cyanobacteria are known to form intense blooms, which can be caused by anthropogenic influences in eutrophic fresh waters (Wiegand and Pflugmacher, 2005). Of particular concern are the hepatotoxic secondary metabolites called microcystins (MCs). Among the more than 70 structural analogues of MCs identified, MC-LR and MC-RR are the most studied types produced by *Microcystis* (Sivonen and Jones, 1999). Most MC are cell bound (Kaebernick and Neilan, 2001), thus intracellular MC levels are typically high and may harm organisms that feed on the toxic cyanobacteria (Codd, 1995; Rohrlack et al., 2001; Lürling, 2003; Juhel et al., 2006; Smith and Haney, 2006; Trubetskova and Haney, 2006). Studies on MC production and related environmental parameters have provided some clues to the regulation and function of these toxins (Sivonen and Jones, 1999; Oh et al., 2000).

Herbivorous zooplankters have different physiological characteristics throughout their life stages, and variation in these characteristics is a sensitive indicator of environmental stress (Krebs, 1985). Previous studies have shown that different zooplankton responses to phosphorus contents, high temperature, or toxic substances throughout their life cycles were associated with growth, feeding efficiency, and swimming ability (DeMott, 2003; Nandini et al., 2004; Christensen et al., 2005). Physiological developmental processes of zooplankton, which involve the secretion of chemical signals, may affect the release of infochemicals as well (Larsson and Dodson, 1993).

Recently, induced defenses mediated by the release of chemical signals from zooplankton have been observed as increased toxin production in several toxic *Microcystis aeruginosa* strains (Jang et al., 2003) and the inducible defenses by toxic cyanobacteria depend on the zooplankton density and infochemical concentrations (Jang et al., 2007). Several examples of induced defenses in phytoplankton include that the colony formation of coenobial green algae (*Scenedesmus*) may depend on the age, size, mass, or weight of zooplankton as well (Lampert et al., 1994; Ha et al., 2001). However, it is not tested whether this induced defense depends on zooplankton developmental stages. It is critical to test the change of MC production in *M. aeruginosa* strains when exposed to different stages of herbivorous zooplankton, because different feeding activity and chemical release from each developmental stage of zooplankton could affect cyanobacteria. In this study, we evaluated the MC production in four toxic *M. aeruginosa* strains in response to indirect exposure to different developmental stages (e.g., neonate/juveniles and adult) of two zooplankton species (*Daphnia magna* Straus and *Moina macrocopa* Leydig) to test the hypothesis that *M. aeruginosa* produces higher concentrations of MC when exposed to adult zooplanktons compared to neonate or juvenile zooplankton.

2. Materials and methods

2.1. Cyanobacteria and zooplankton cultivation

Four strains of *M. aeruginosa* (Kützing) Lemmermann [strains 89, 98, 103 and 107; Microbial Culture Collection, National Institute for Environmental Studies (NIES), Tsukuba, Japan] were used for this study. All four strains are axenic and monoclonal (Kasai et al., 2004). Each strain was axenically grown in batch culture in CT medium (adjusted pH 8.2; medium composition by Jang et al., 2004) at 27 °C in an incubator (MLR-351H, SANYO, Japan) with a light–dark (LD) regime of 16:8 (irradiance, 120 μmol $m^{-2} s^{-1}$). The composition of CT medium was 15 mg $Ca(NO_3)_2 \cdot 4H_2O$, 10 mg KNO_3, 5 mg β-Na_2glycerophosphate $\cdot 5H_2O$, 4 mg $MgSO_4 \cdot 7H_2O$, 0.01 μg vitamin B_{12}, 0.01 μg bioton, 1 μg thiamine HCl, 0.3 ml of PVI metals (comprising 19.6 mg $FeCl_3 \cdot 6H_2O$, 3.6 mg $MnCl_2 \cdot 4H_2O$, 2.2 mg $ZnSO_4 \cdot 7H_2O$, 0.4 mg $CoCl_2 \cdot 6H_2O$, 0.25 mg $Na_2MoO_4 \cdot 2H_2O$, and 100 mg $Na_2EDTA \cdot 2H_2O$, and 100 ml distilled water), 40 mg *N*-tris (hydroxymethyl)-methyl-3-aminopropanesulfonic acid (TAPS), and 99.7 ml distilled water (Kasai et al., 2004). *M. aeruginosa* cells in their exponential growth phase were used in our experiments.

Two developmental stages of *D. magna* (juvenile stage, 2–3 days old, mean length ± SD, 1.5 ± 0.2 mm, mean dry weight ± SD, 0.21 ± 0.07 mg; non-egg-bearing adult stage, 7–8 days old, 2.3 ± 0.4 mm, 0.35 ± 0.09 mg) and *M. macrocopa* (neonate stage, within 1 day after hatching, 0.2 ± 0.03 mm, 1.6 ± 0.2 μg; non-egg-bearing adult stage, 3–4 days old, 1.2 ± 0.1 mm, 0.09 ± 0.03 mg) were obtained

from stock cultures that were maintained under laboratory conditions at NIES. *Scenedesmus* ($\sim \times 10^3$ cell ml^{-1}) were supplied as a food source to stock cultures of *D. magna* and *M. macrocopa* in 2 l of dechlorinated water, which were maintained at 20 °C (light–dark regime of 16:8, irradiance 120 μmol m^{-2} s^{-1}). Neonates of *M. macrocopa* were obtained from stock cultures that were maintained at 27 °C (light–dark regime of 16:8, irradiance 120 μmol m^{-2} s^{-1}) to increase the hatching rate.

2.2. Indirect zooplankton exposure experiment

To obtain zooplankton culture media filtrate (ZCMF) containing dissolved chemicals released from zooplankton, 200 non-egg-bearing adults of *D. magna*, 200 non-egg-bearing adults of *M. macrocopa*, and 200 juveniles of *D. magna* were incubated separately for 48 h in 2 l of dechlorinated water, with *Scenedesmus* provided above the concentration at which zooplankton are food limited ($\sim \times 10^3$ cells ml^{-1}). Two hundred neonates of *M. macrocopa* were incubated for 24 h in 2 l of dechlorinated water without any food. After removal of the zooplankton by GF/C filtration, each water sample was passed through a Nucleopore filter (0.2 μm pore size; Whatman, Tokyo, Japan) in a sterilized room to remove algal cells, bacteria, and other particulates.

To determine the cyanobacterial response to indirect exposure to the two developmental stages of these zooplankton species, the four *M. aeruginosa* strains were inoculated separately in flasks with 300 ml of CT medium containing 150 ml ZCMF (50% of the total volume of culture media was zooplankton filtrate and the reminder was standard CT media) of each developmental stage for *D. magna* (DJCMF and DACMF, juvenile- and adult-stage culture media filtrate, respectively) and *M. macrocopa* (MNCMF and MACMF, neonate- and adult-stage culture media filtrate, respectively). For each strain, triplicates of each treatment (3×2 zooplankton species $\times 2$ developmental stages $\times 6$ days) as well as a control containing no ZCMF (3×7 days including day 0) were prepared (a total of 93 flasks per strain). The flasks were incubated at a constant temperature of 27 °C and a light intensity of 120 μmol m^{-2} s^{-1} using continuous cool white fluorescent lamps (light–dark regime, 16:8 h). The flasks were shaken four times a day to mix the cyanobacterial culture. At each day, three control flasks and three flasks from each treatment (a total of 15 flasks per day per strain) were chosen randomly for analysis.

Cyanobacterial biomass (as a dry weight), intracellular MC production, and extracellular MC production were analyzed daily until day 6. Nutrient concentrations (NO_3–N, NO_2–N, NH_4–N, and PO_4–P) were analyzed on days 0 and 6 from 20 ml filtrate water of each flask. 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 the standard methods (APHA et al., 1995). Cyanobacterial biomass was assessed by measuring freeze-dried weight after filtration and centrifugation from each flask. For the analysis of intracellular MC, cells were harvested by centrifugation at 12,000*g* at 4 °C, freeze-dried, weighed on a balance (PB303-S Delta Range, Mettler, Toledo, OH, USA), and then stored at −70 °C until MC analysis. To obtain extracellular MC, the GF/C filtered water was passed through a 1.431 g Oasis® HLB 1-cc extraction cartridge (Waters, Milford, MA, USA) and then the cartridge samples were stored at 4 °C until MC analysis. Preliminary analyses of both types of ZCMF from the same zooplankton stocks as those used in the experiment contained no MC for the 6 days prior to the experimental exposure to cyanobacteria. The dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorus (DIP) concentrations of zooplankton-cultured waters were 0.7–1.6 mg l^{-1} and 0.03–0.09 mg l^{-1} ($n = 4$), respectively.

2.3. Microcystin analysis

Purification and analysis of MC were performed using the methods developed by Harada et al. (1988) and Oh et al. (2000). Intracellular MCs were extracted from freeze-dried cyanobacterial cells twice with 30 ml of 5% acetic acid (v/v) for 16 h while shaking at 140 rpm. The extract was centrifuged at 12,000*g*, and then the supernatant was applied to an extraction cartridge (Oasis® HLB 1-cc, Waters). The supernatant for intracellular MC and extracellular MC was eluted with 100% 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 mm × 150 mm, 5.0, Shiseido, Tokyo, Japan) reverse-phase column; the mobile phase was methanol and 0.05 mol l^{-1} phosphate buffer (58:42, pH 3.0). The MCs were

identified by their UV spectra and retention times and by spiking the sample with purified standards of MC-LR and MC-RR (Wako, Richmond, VA, USA). The MC peaks were isolated and identified according to their mass spectra. The detection limits for intracellular MC and extracellular MC were $0.1\,\mu g\,g^{-1}$ dry weight (DW) and $0.1\,\mu g\,ml^{-1}$, respectively. Each analysis was performed in duplicate. In this study, the sum of MC-LR and MC-RR is referred to as 'MC'. The intracellular MC concentration is expressed as $\mu g\,g^{-1}$ DW and the extracellular MC concentration is expressed as $\mu g\,ml^{-1}$.

2.4. Statistics

Differences in cyanobacterial biomass, intracellular MC concentration, and extracellular MC concentration among the control and ZCMF treatments over time were assessed using a repeated-measurement analysis of variance (rm-ANOVA), and *post hoc* comparisons were performed using Tukey multiple tests. Differences in intracellular MC and extracellular MC concentrations among the control and ZCMF treatments on the peak day (the day of highest concentrations) were assessed using a one-way ANOVA; when values demonstrated a significant difference, *post hoc* Tukey multiple comparison tests were used to explore differences in intracellular MC and extracellular MC among repeated means (SPSS Release 12.0; SPSS inc., Chicago, IL, USA). The data on day 0 were excluded from the analysis.

3. Results

Exposure to ZCMF stimulated the growth of all four cyanobacterial strains (Figs. 1A–D, 2A–D), although the biomasses were not significantly different among all ZCMF treatments (rm-ANOVA followed by *post hoc* Tukey test, $p > 0.05$). A temporal decrease of DIN (from 31 to $32\,mg\,l^{-1}$ on day 0 to 24–29 $mg\,l^{-1}$ on day 6) and a temporal increase of DIP (from 1.0 to $1.6\,mg\,l^{-1}$ on day 0 to 1.0–2.8 $mg\,l^{-1}$ on day 6) were observed in both the control and zooplankton treatments for all four strains.

For all four strains, the intracellular MC concentrations significantly differed among the control and neonate/juvenile or adult zooplankton treatments (Figs. 1E–H, 2E–H). Over the course of the experiment, intracellular MC concentrations were significantly higher in adult *Daphnia* treatment (DACMF) than in juvenile *Daphnia* treatment (DJCMF) for strains 89 and 107 (rm-ANOVA and *post hoc* Tukey test, $p < 0.001$), and the intracellular MC concentrations were significantly higher in adult *Moina* treatment (MACMF) than in neonate *Moina* treatment (MNCMF) for three strains (rm-ANOVA and *post hoc* Tukey test; $p = 0.010$, 0.009, and 0.005 for strains 89, 98, and 107, respectively). Intracellular MC production peaked on days 3–4 in adult zooplankton treatments and on days 2–3 in juvenile/neonate treatments. Upon exposure to the zooplankton treatments, each strain showed a different extent of increased intracellular MC production (Figs. 1 and 2). Throughout the experiment, strain 98 produced markedly lower intracellular MC concentrations compared with those of other strains.

Although very low concentrations of extracellular MC (mean, 9.9–737.6 $\mu g\,ml^{-1}$) were released compared with the intracellular MC concentrations, the temporal patterns of extracellular MC production in the zooplankton treatments were similar to those of intracellular MC (Figs. 1I–L, 2I–L). The strains showed significantly higher MC production in the adult zooplankton treatments compared to the juvenile/neonate treatments (rm-ANOVA followed by *post hoc* Tukey test, $p < 0.036$) in all cases except that of the strain 103 in the both of *Daphnia* treatments ($p = 0.113$). Extracellular MC production peaked on days 2–3. Strain 89 showed the greatest increase in extracellular MC production in adult zooplankton treatments compared to the other strains, and this strain's extracellular MC concentrations were 3–4 times higher than those of the control or juvenile/neonate treatments on the peak day (Figs. 1 and 2).

Assuming that the temporal patterns of MC production in the four *Microcystis* strains are similar upon indirect exposure to either zooplankton species, a unimodal curve can be drawn. Examination of unimodal curves for the patterns of intracellular MC and extracellular MC concentrations for all four strains in indirect zooplankton exposure experiment showed that the cyanobacteria showed peak MC production on day 3 (Fig. 3). The intracellular MC concentrations in the adult zooplankton treatments were significantly higher than those in the control or juvenile/neonate treatments (Fig. 3A and B; univariate ANOVA, $p < 0.001$). The intracellular MC concentration in juvenile *Daphnia* treatment was also significantly higher than that of

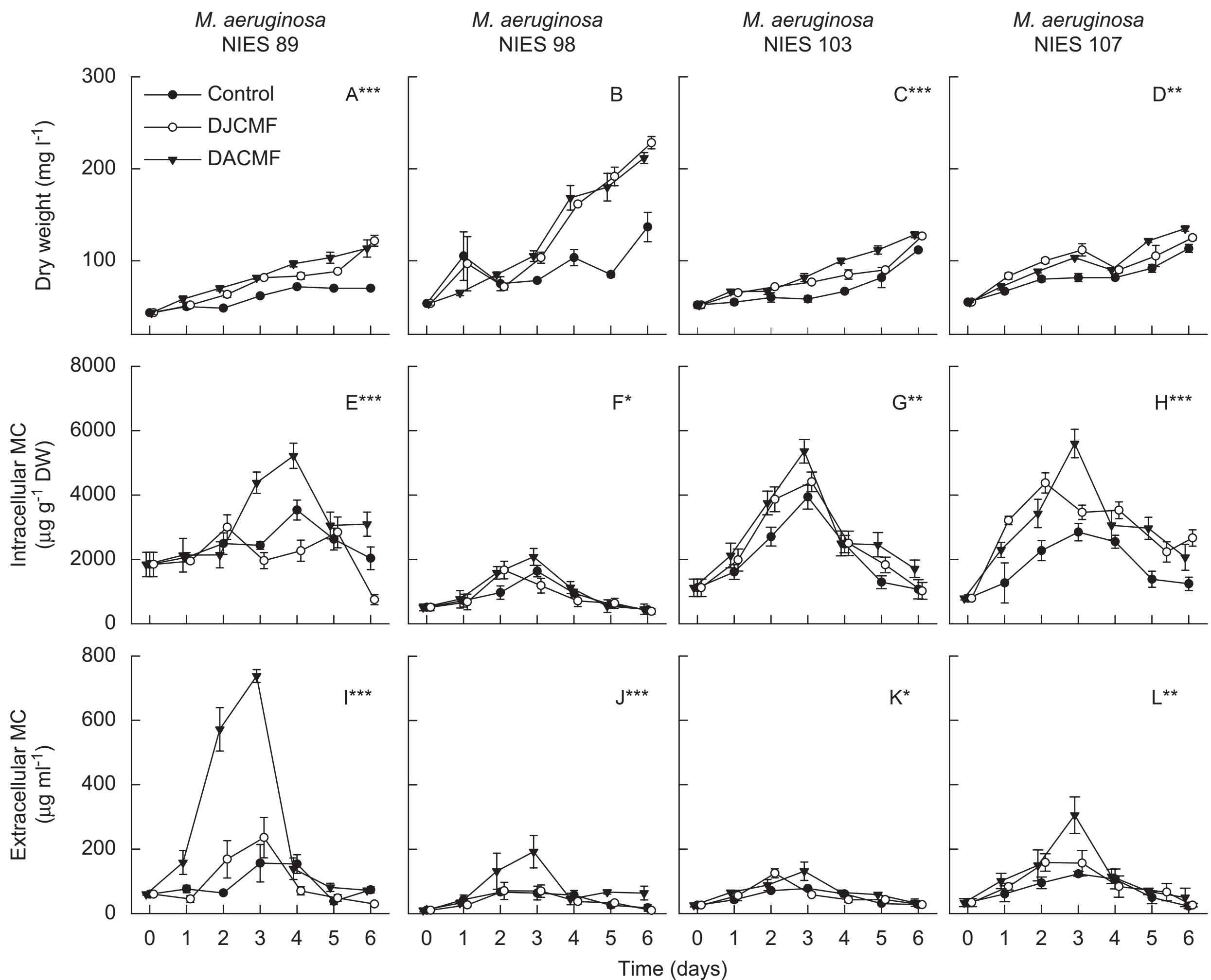

Fig. 1. Changes in dry weight, intracellular microcystin (MC) concentrations, and extracellular MC concentrations of four *Microcystis aeruginosa* strains (strains 89, 98, 103, and 107) from the control and *Daphnia* treatments over 6 days. Culture media filtrates from juvenile and adult *Daphnia magna* were used for two *Daphnia* treatments (DJCMF and DACMF, respectively). Values are mean ± SE ($n = 3$). Significant differences among controls and treatments are indicated by $^*p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$.

the control ($p<0.001$), whereas that in neonate *Moina* treatment was similar to the control ($p = 0.994$). The peak intracellular MC production in adult *Daphnia* treatment (mean value of 4239 $\mu g\,g^{-1}$ DW) was higher than that in adult *Moina* treatments (mean value of 3713 $\mu g\,g^{-1}$ DW).

The extracellular MC concentrations in adult zooplankton treatments were significantly higher than those of the control, juvenile/neonate treatments (Fig. 3C and D; univariate ANOVA, $p<0.001$), whereas those in the juvenile/neonate treatments were not significantly higher than in the control ($p = 0.499$ for DJCMF; $p = 0.889$ for MNCMF). The peak extracellular MC production in adult *Daphnia* treatment (mean value of 374 $\mu g\,ml^{-1}$) was higher than that in adult *Moina* treatments (mean value of 278 $\mu g\,ml^{-1}$).

4. Discussion

This study is the first to provide evidence of greater MC production by cyanobacteria after exposure to infochemicals from adult zooplankton compared to those from neonate/juvenile zooplankton. This developmental stage-dependent result provides good support for our earlier study, in which we found differences in intracellular MC production caused by exposure to three zooplankton species (e.g., *D. magna*, *Daphnia. Pulex*, and *M. macrocopa*; Jang et al., 2003). In this study, the

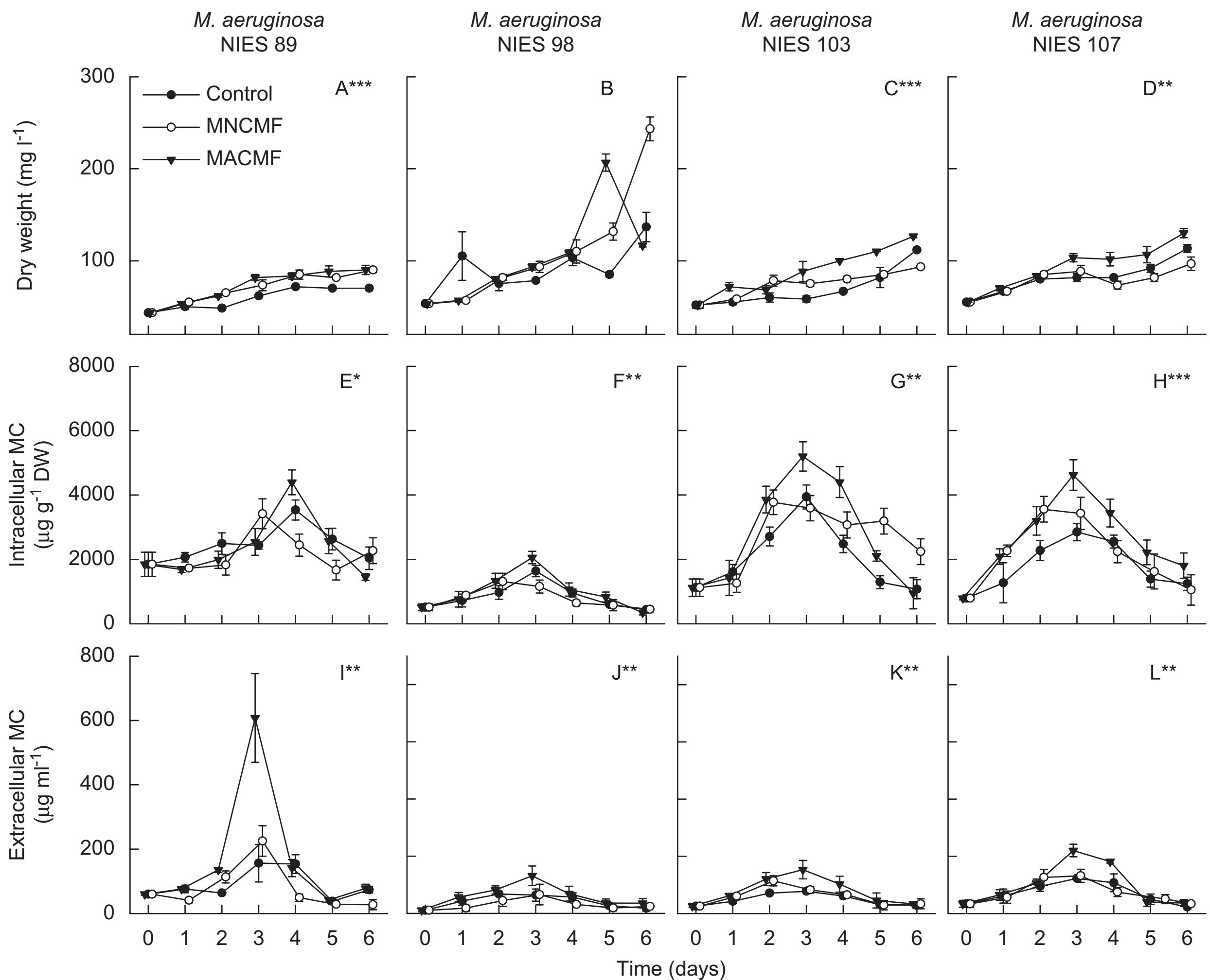

Fig. 2. Changes in dry weight, intracellular microcystin (MC) concentrations, and extracellular MC concentrations of four *Microcystis aeruginosa* strains (strains 89, 98, 103, and 107) from the control and *Moina* treatments over 6 days. Culture media filtrates from neonate and adult *Moina macrocopa* were used for two *Moina* treatments (MNCMF and MACMF, respectively). Values are mean$\pm$SE ($n = 3$). Significant differences among controls and treatments are indicated by $*p<0.05$, $**p<0.01$, and $***p<0.001$.

higher intracellular MC and extracellular MC production in the adult zooplankton treatments might be explained by the fact that adult zooplankton excrete more chemical signals than equal numbers of smaller juveniles or neonates, since the differences in masses between adults and juveniles/neonates would be very large, usually more than 10-folds. Growth of zooplankton is defined as the net difference between the catabolic and anabolic metabolism (Becker et al., 2000), and the changes in physiological characteristics, body size (e.g., body mass), and feeding habit or rate at different developmental stages might be related to the induction of infochemicals.

Higher intracellular MC and extracellular MC production were observed in the adult *Daphnia* treatment (DACMF) than in the adult *Moina* treatment (MACMF). Compared to *M. macrocopa*, *D. magna* has larger body size (body mass) and a higher feeding rate, which in turn are attributed to higher infochemical production (DeMott, 2003). In experiments using direct exposure to zooplankton (Jang et al., 2007), peak intracellular MC production was also higher in adult *Daphnia* than that in adult *Moina* treatments.

In agreement with Kasai et al. (2004), each monoclonal *M. aeruginosa* strain showed different MC production capabilities when exposed to

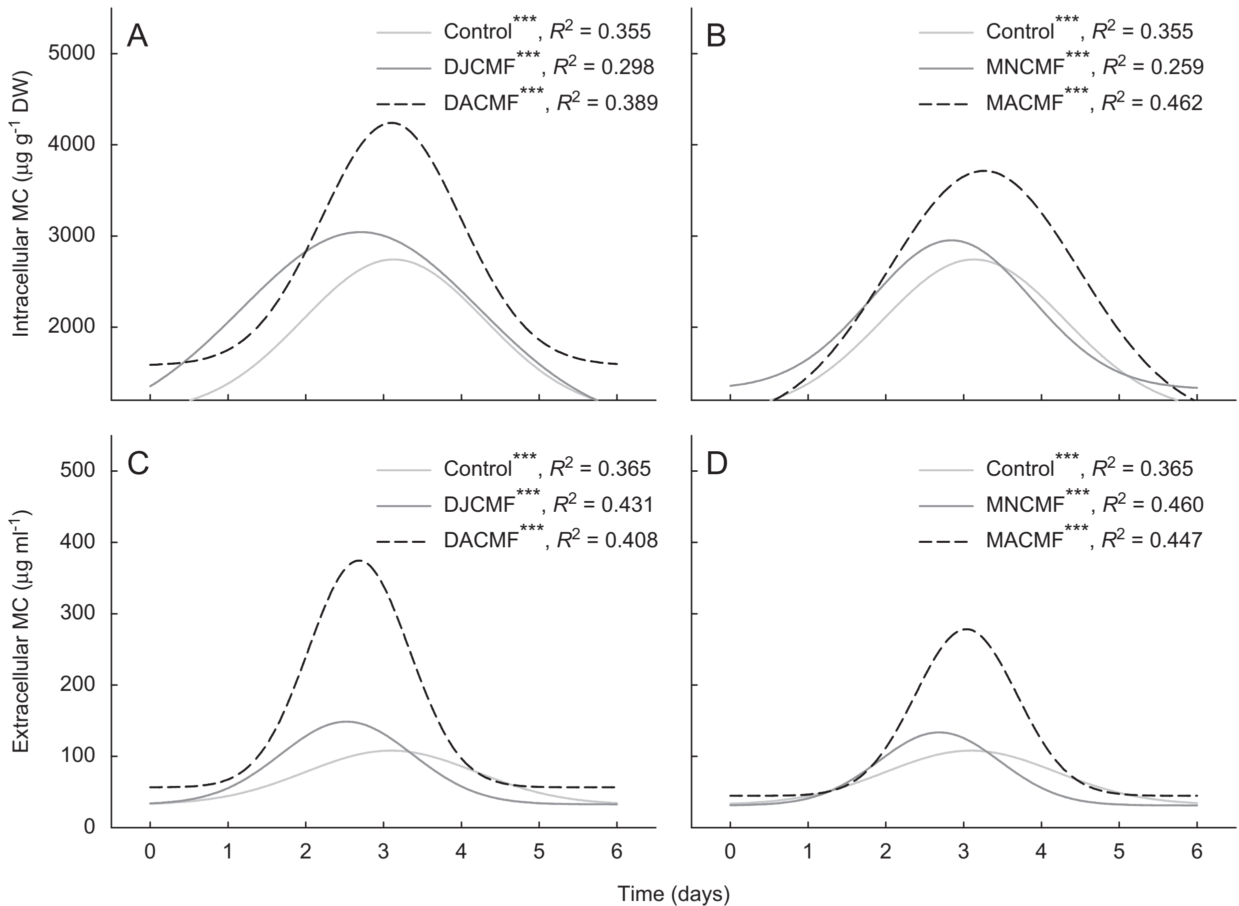

Fig. 3. Unimodal curve of the concentrations of intracellular microcystins (MC) and extracellular MC in indirect exposure experiments over time. The curves represent the average of mean MC concentrations for each strain ($n = 4$). The significance of each curve is indicated by $*p<0.05$, $**p<0.01$, and $***p<0.001$.

zooplankton treatments in our study. Toxic strains contain the microcystin synthetase (*mcy*) gene, which is involved in MC synthesis (Kaebernick and Neilan, 2001). Uncertainties remain regarding the mechanism of extracellular MC release, although Rapala et al. (1997) reported that cell death and lysis release intracellular cyanobacterial toxins into the surrounding waters, and the concentration of extracellular toxins increases as blooming toxic cyanobacteria age in eutrophic waters. In our study, compared with those of neonate/juvenile treatments, extracellular MC increased with adult zooplankton treatments in both *Daphnia* and *Moina* treatments, similarly to the patterns of intracellular MC. This finding suggests that cyanobacteria release MC into the extracellular environment as an induced defense against zooplankton, and that this release is triggered by infochemcials produced by the zooplankton. Extracellular MC concentrations also differed among strains, with strain 89 showing the greatest relative increase upon exposure to adult zooplankton treatments compared to other strains.

In conclusion, this study revealed the potential impacts of infochemicals released from herbivorous zooplankton at different developmental stages, mediated with physiological characteristics, body masses, or feeding habits on MC production in *Microcystis*. There is more juvenile zooplankton than adult in lake, and the juveniles may contribute more to the infochemical pools in lake; however, considering equal numbers of them, adult zooplankton produce more chemical signal than smaller juvenile/neonate, therefore more increased intracellular MC and extracellular MC production were observed in adult ZCMF rather than in neonate/juvenile zooplankton. Further work is needed to establish the composition of zooplankton infochemicals

and whether *Microcystis* recognizes the different developmental stages of zooplankton by way of chemical cues.

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