

Changes in microcystin production in cyanobacteria exposed to zooplankton at different population densities and infochemical concentrations

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Abstract

We investigated microcystin (MC) production by four cyanobacterial strains (three *Microcystis aeruginosa* and one *Planktothrix agardhii*) in response to different grazer densities (direct exposure: zero, two, four, or eight individuals per 300 mL) and infochemical concentrations (indirect exposure: 0%, 10%, 25%, and 50%) of *Daphnia magna* and *Moina macrocopa*. MC production increased after direct exposure to both zooplankton species and was higher with increasing concentration of infochemicals. This MC production was significantly different among the control and three zooplankton treatment levels. Upon direct and indirect exposure of cyanobacteria to zooplankton, intracellular MC peaked on days 3 and 4. In most cyanobacterial strains, the peak MC contents were significantly higher in direct treatment with the highest zooplankton density and in indirect treatment with the highest concentration of zooplankton culture media filtrate than with treatments with the lowest density and concentration, respectively. Extracellular MC concentrations were much lower than intracellular ones, but both showed similar temporal patterns over the course of the experiment. Cyanobacteria directly exposed to *Daphnia* released greater amounts of extracellular MC than did those exposed to *Moina*. This is the first study to provide evidence of an induced defense of increased MC production by cyanobacteria in response to increasing zooplankton grazer density and increased concentrations of infochemicals released by zooplankton. In addition to the induction of tolerance in *Daphnia* to toxic *Microcystis*, we discuss how these reciprocal defenses may explain the coexistence of zooplankton and toxic cyanobacteria in eutrophic freshwaters.

Under favorable conditions in eutrophic fresh, brackish, and marine waters, cyanobacteria can proliferate rapidly to form blooms and scums. Of particular concern are the cyanobacterial genera *Microcystis*, *Anabaena*, *Aphanizomenon*, *Planktothrix*, and *Nostoc*, which may produce a wide range of hepatotoxic secondary metabolites called microcystins (MCs; Sivonen and Jones 1999). Because most MCs are cell bound, intracellular MC levels are typically high and may therefore harm organisms that feed on the toxigenic cyanobacteria (Kaebernick et al. 2000); these toxigenic properties allow the cyanobacteria to escape grazing pressure and gain a competitive advantage in eutrophic waters (DeMott et al. 1991). Several studies on

cyanotoxin production and related environmental parameters, including food-web components, have provided some clues to the regulation and function of these toxins (Kaebernick and Neilan 2001). Toxic strains possess *mcy* genes involved in MC synthesis, although the genes may fragment or mutate and thus become nonfunctional (Tillett et al. 2001).

Prey organisms defend themselves against predators through various mechanisms. Inducible defenses can be important ecological factors, with both direct and indirect effects at the community level. Examples of inducible defenses are refuge use, reduced activity, life-history changes, toxin production, colony formation, and development of helmets or spines. Inducible defenses are highly influenced by information-conveying chemicals (Dicke and Sabelis 1988). Recent observations indicate that induced chemical defenses are widespread and occur in plants and animals from terrestrial, marine, and freshwater habitats (Tollrian and Harvell 1999), including planktonic systems (e.g., infochemical-mediated larger coenobia formation in *Scenedesmus* against the presence of *Daphnia*; van Donk et al. 1999), plant–insect systems (e.g., herbivory-induced plant production of volatiles that attract carnivorous arthropods; Dicke 1999), ciliated protozoal systems (e.g., defensive changes in shape of *Euplotes* against predator-derived kairomones; Kuhlmann et al. 1999), zooplankton systems (e.g., defensive polymorphism in

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rotifers in response to kairomones produced by various predators, such as *Asplanchna*, copepods, and the interference competitors cladocerans; Gilbert 1999), and zooplankton–fish systems (e.g., diel vertical migration or depth-selection behavior of zooplankton when exposed to fish kairomones; Meester et al. 1999).

A well-studied example of induced defenses in freshwater algae is the morphological changes observed in coenobial green algae and filamentous blue-green algae in water containing *Daphnia* (Fiałkowska and Pajdak-Stós 1997; Lüring and van Donk 2000). Chemicals in water that contains zooplankton may function as infochemicals, and induced antipredator morphological defenses have been shown to depend on consumer density and to be triggered by infochemical cues (Lampert et al. 1994; van Donk et al. 1999), although the identity of these chemicals is still under debate.

The ecological interactions between cyanobacteria and zooplankton have received considerable attention. Cyanobacteria are inadequate food sources for zooplankton owing to the existence of grazing-resistant forms, such as filamentous or colonial (DeMott et al. 2001), and their lack of essential fatty acids or lipids (DeMott and Müller-Navarra 1997); these characteristics mean that cyanobacteria could have severe effects on zooplankton growth and reproduction (von Elert and Wolffrom 2001). Moreover, cyanobacterial toxins can impair zooplankton feeding behavior (Ghadouani et al. 2004), reduce their filtering rates (DeMott et al. 1991), and even kill zooplankton. Some zooplankton, however, appear to have evolved physiological and behavioral traits to survive in the presence of certain toxic cells. Recent observations suggest that zooplankton populations may adapt to tolerate toxic cyanobacteria (Sarnelle and Wilson 2005), and that this trait can be transferred from mother to offspring (Gustafsson et al. 2005), indicating that it has a genetic basis.

Several field surveys have revealed that the dominance of the noxious cyanobacterium *Microcystis aeruginosa* is associated with the presence of consumers. Zebra mussels, exotic species in the Great Lakes, promote the dominance of *Microcystis* in phytoplankton communities in lakes with relatively low total phosphorus levels (Raikow et al. 2004). Studies have shown that increased toxin production in several *M. aeruginosa* strains is an induced defense mediated by physical contact associated with feeding or by chemical cues from herbivorous zooplankton or phytoplanktivorous fish (Jang et al. 2003, 2004). However, it is still unclear whether this induced antipredator defense depends on zooplankton density or infochemical concentration, or both. Toxic algal blooms are not limited to fresh waters, however, and they are a problem in shallow coastal marine environments as well. Thus, from general scientific and resource-management perspectives, it is critical that we improve our understanding of the ecological implications of herbivore-induced MC production in cyanobacteria.

In a previous study, we found that potentially toxic cyanobacteria increase their toxin production in response to the presence of zooplankton, as an induced defense mediated by the release of infochemicals from the zooplankton (Jang et al. 2003). In the present study, we

investigated MC production by four strains of two cyanobacteria species (three strains of *M. aeruginosa* and one strain of *Planktothrix agardhii*) in response to different levels of direct or indirect exposure to herbivorous zooplankton (*Daphnia magna* and *Moina macrocopa*). Using zooplankton exposure experiments, we investigated whether toxin production by cyanobacteria depends on grazing activity (direct) or infochemical concentration (indirect), or both. Our aim was to test the hypothesis that MC production by cyanobacteria increases in response to increasing zooplankton grazer density and to infochemicals released by the zooplankton.

Materials and methods

Cyanobacteria and zooplankton cultivation—Three strains of *M. aeruginosa* (Kützing) Lemmermann (strains 88, 98, and 107 from the Microbial Culture Collection, National Institute for Environmental Studies [NIES], Ibaraki, Japan) and one strain of *P. agardhii* Anagnostidis et Komárek (strain 1264, from NIES) were used. 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) at 27°C in an incubator with a light:dark (LD) regime of 16:8 (irradiance, 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The composition of CT medium was 15 mg of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 10 mg of KNO_3 , 5 mg of $\beta\text{-Na}_2$ glycerophosphate $\cdot 5\text{H}_2\text{O}$, 4 mg of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 μg of vitamin B₁₂, 0.01 μg of biotin, 1 μg of thiamine HCl, 0.3 mL of PVI metals (comprising 19.6 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 3.6 mg of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 2.2 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 mg of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.25 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 100 mg of Na_2 ethylenediaminetetraacetic acid $\cdot 2\text{H}_2\text{O}$, and 100 mL of distilled water), 40 mg of *N*-tris(hydroxymethyl)-methyl-3-aminopropanesulfonic acid, and 99.7 ml of distilled water (Kasai et al. 2004). Cyanobacteria in their exponential growth phase were used in our experiments. Non-egg-bearing adults of *D. magna* Straus (mean length \pm SD, 2.3 \pm 0.4 mm; mean dry weight \pm SD, 0.43 \pm 0.11 mg) and *M. macrocopa* Leydig (1.2 \pm 0.1 mm; 0.09 \pm 0.03 mg) were obtained from stock cultures that were maintained under laboratory conditions at NIES with *Scenedesmus* as food.

Direct zooplankton exposure experiment—To investigate the cyanobacterial response to direct zooplankton exposure, the four strains of cyanobacteria were separately cultured in flasks with 300 mL of CT medium (Kasai et al. 2004) and two, four, or eight individuals of *D. magna* (DT1, DT2, and DT3, respectively) or *M. macrocopa* (MT1, MT2, and MT3, respectively) per flask. The calculated biomass (as dry weight) of *D. magna* in each treatment was 0.86, 1.72, and 3.44 mg in DT1, DT2, and DT3, respectively. That of *M. macrocopa* was 0.18, 0.36, and 0.72 mg in MT1, MT2, and MT3, respectively. Triplicates of each treatment (3 \times 3 levels of zooplankton density \times 2 zooplankton species \times 6 d), as well as a control containing no zooplankton (3 \times 7 d including day 0) were used (a total of 129 flasks) for each strain. The flasks were incubated in a growth chamber (27°C, 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 16:8 LD regime). 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.

Cyanobacterial biomass, zooplankton survival rates, intracellular MC, and extracellular MC were analyzed daily until day 6. Nutrient ($\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$, $\text{NH}_4\text{-N}$, and $\text{PO}_4\text{-P}$) concentrations were analyzed on days 0 and 6. The water samples for $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$, $\text{NH}_4\text{-N}$, and $\text{PO}_4\text{-P}$ were passed through a GF/F filter and then measured according to standard methods (APHA et al. 1995). Cyanobacterial biomass was monitored by measuring freeze-dried weight. Live zooplankton were counted each day, and survival rates were calculated as percentages. For the analysis of intracellular MC, cells were harvested by centrifugation at $12,000 \times g$ at 4°C , freeze-dried, weighed on a balance (PB303-S Delta Range, Mettler), and then stored at -70°C until analysis. To obtain extracellular MC, the GF/C filtered water was passed through a 1.431-g Oasis HLB 1-cc extraction cartridge (Waters), and then the cartridge samples were stored at 4°C until MC analysis.

Indirect zooplankton exposure experiment—To obtain zooplankton culture media filtrates (ZCMFs) containing dissolved chemicals released from zooplankton, 200 non-egg-bearing adult *D. magna* and 300 non-egg-bearing adult *M. macrocopa* were incubated for 4 and 2 d, respectively, in 4 liters of dechlorinated water with *Scenedesmus* above the concentration at which zooplankton are food limited (i.e., 10^3 cells mL^{-1}). After removal of the zooplankton by GF/C filtration, the water was passed through 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 zooplankton exposure, the four strains of cyanobacteria were separately cultured in flasks with 300 mL of CT medium and three levels of *D. magna* filtrate (DCMF1, DCMF2, and DCMF3: 10%, 25%, and 50% of the total volume of culture media was *D. magna* filtrate, respectively, and the remainder was standard CT media) and *M. macrocopa* filtrate (MCMF1, MCMF2, and MCMF3: 10%, 25%, and 50%, respectively). Triplicates of each treatment as well as a control containing no ZCMF (a total of 129 flasks) were used for each strain. The culture methods and determination of nutrient concentrations, cyanobacterial biomass, intracellular MC, and extracellular MC were the same as for the direct zooplankton exposure experiment described above. The DIN (dissolved inorganic nitrogen) and DIP (dissolved inorganic phosphorus) concentrations of filtered zooplankton-cultured waters were 0.7–1.3 mg L^{-1} and 0.1–0.15 mg L^{-1} ($n = 4$), respectively.

Microcystin analysis—Purification and analysis of MC were performed using the methods developed by Harada et al. (1988). In this study, the sum of MC-LR and MC-RR is referred to as “MC”. Intracellular MC was extracted twice from freeze-dried cyanobacterial 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 $12,000 \times g$, and the supernatant was applied to a 1.431-g Oasis HLB 1-cc extraction cartridge (Oasis, Waters). The supernatants for

intracellular and extracellular MCs 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 mm \times 150 mm, 5.0, Shiseido) reverse-phase column, and the mobile phase was methanol, 0.05 mol L^{-1} 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). The MC peaks were isolated and identified according to their mass spectra. The detection limits for intracellular and extracellular MCs were 0.1 $\mu\text{g g}^{-1}$ dry weight (DW) and 0.1 $\mu\text{g mL}^{-1}$, respectively. Each analysis was performed in duplicate. The intracellular MC concentration was expressed as $\mu\text{g g}^{-1}$ DW and the extracellular MC concentration as $\mu\text{g mL}^{-1}$ of water samples.

Statistics—Differences in cyanobacterial biomass, intracellular MC, and extracellular MC among the control and the zooplankton treatments over time were assessed by using a repeated-measurement analysis of variance (RM-ANOVA), and post hoc comparisons were performed using Tukey multiple tests. Changes in the intracellular MC and extracellular MC between *Daphnia* and *Moina* treatments over time were also assessed by RM-ANOVA. Differences in intracellular MC and extracellular MC concentrations among the control and zooplankton treatments on the peak day were assessed by one-way ANOVA. When values demonstrated a significant difference, a post hoc Tukey multiple comparison test at the peak day was used to explore differences in intracellular MC and extracellular MC concentrations among repeated means (SPSS Release 12.0; SPSS). Data gathered on day 0 were excluded from the analysis.

Results

Direct-exposure experiment—Overall, the cyanobacterial biomass remained constant or increased slightly over the course of the experiment, with the exception of the biomass of *Microcystis* strain 98 in the highest *D. magna* treatment (DT3), which decreased (Figs. 1A–D, 2A–D). For *Microcystis* strains 88 and 107 and *Planktothrix* strain 1264, the patterns of zooplankton survival were similar among the *Daphnia* and *Moina* treatments: the inoculated animals started to die on day 1, with more than 80% mortality recorded on day 2 (Figs. 1E–H, 2E–H). However, some zooplankton in flasks with *Microcystis* strain 98 were alive to the end of the experiment. Similar nutrient concentrations were observed for all cyanobacterial strains in the control and *Daphnia* and *Moina* treatments. The DIN concentrations decreased slightly from 32–34 mg L^{-1} on day 0 to 28–32 mg L^{-1} on day 6, whereas DIP concentrations increased slightly from 1.2–5.2 mg L^{-1} on day 0 to 1.7–5.3 mg L^{-1} on day 6.

For all four strains the intracellular MC concentration differed significantly among the control and three zooplankton treatment levels, and intracellular MC levels increased after direct exposure to both zooplankton species

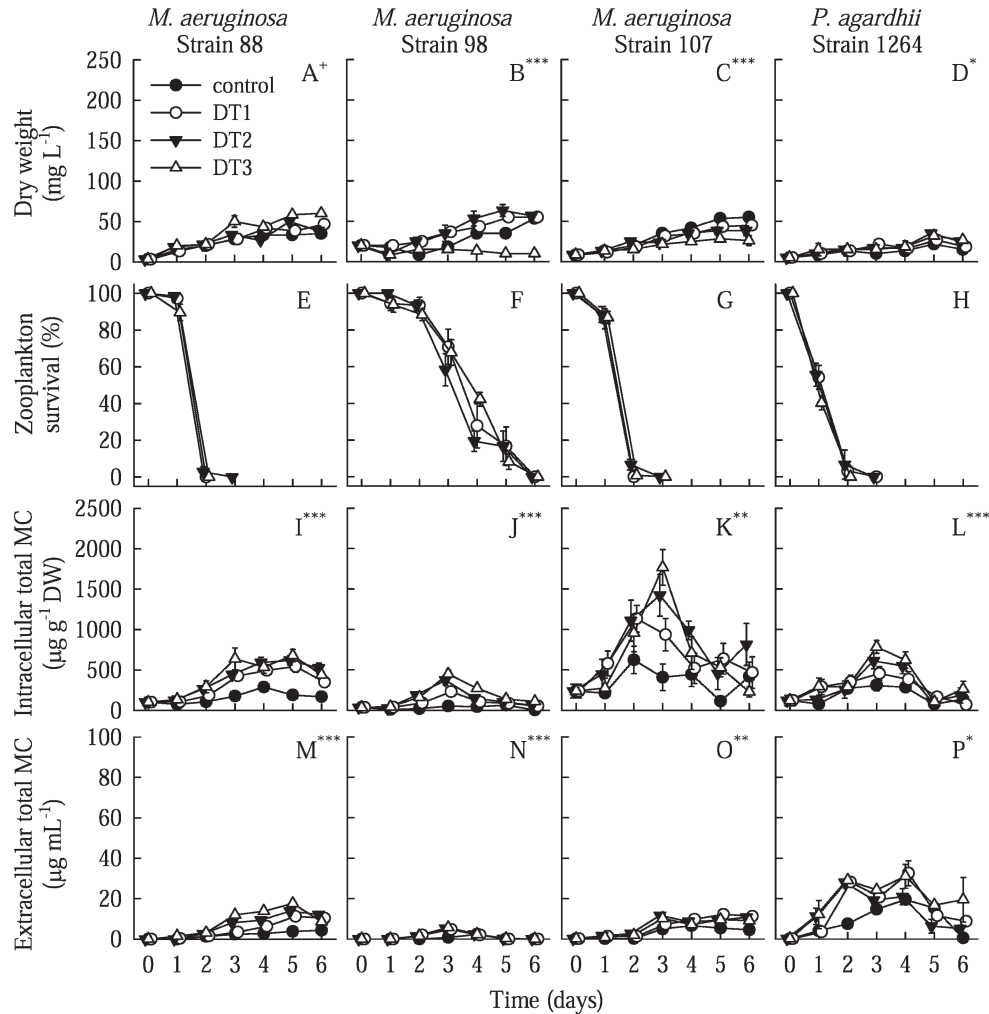


Fig. 1. Changes in cyanobacterial dry weight, zooplankton survival rate, intracellular MC, and extracellular MC when four cyanobacterial strains (three strains of *Microcystis aeruginosa* and one strain of *Planktothrix agardhii*) were directly exposed to *Daphnia magna* at three population densities (two, four, or eight individuals) or to the control. Data are means \pm SE ($n = 3$). Significant differences between controls and treatments on the basis of RM-ANOVA test are indicated by $+p < 0.1$, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

(Figs. 1I–L, 2I–L). Over the course of the experiment the intracellular MC levels were significantly higher in DT3/MT3 than those in DT1/MT1, except in the case of *Microcystis* strains 107 (DT) and 88 (MT; RM-ANOVA and post hoc Tukey test, $p < 0.05$). The intracellular MC levels peaked on day 3, except in the case of *Microcystis* strain 88, for which they peaked on day 4 (MT in Fig. 2) or day 5 (DT in Fig. 1). On the peak day, the MC concentrations of all four strains exposed to DT2/MT2 and DT3/MT3 were significantly higher than those of the control, with the exception of those of *Planktothrix* strain 1264 in treatment MT2 (Table 1). The intracellular MC contents in treatment DT3 were significantly higher than those of DT1 for *Microcystis* strains 98 and 107 and *Planktothrix* strain 1264; the intracellular MC content of *Microcystis* strain 98 in treatment MT3 was significantly higher than in MT1 (Table 1). Of the four strains, *Microcystis* strain 107 showed the greatest increase in intracellular MC production after direct exposure to

zooplankton, and its intracellular MC levels were two to four times higher than those of the control (Figs. 1I–L, 2I–L).

Compared with intracellular MC levels, very low levels of extracellular MC were released by the cyanobacteria (Figs. 1M–P, 2M–P). Over the course of the experiment the extracellular MC levels in treatments DT2/MT2 and DT3/MT3 were significantly higher than those in the control for all four strains (RM-ANOVA test and post hoc Tukey test, $p < 0.05$). Extracellular MC levels peaked at days 3–5; for *Microcystis* strain 88 in the MT treatments, however, extracellular MC levels increased throughout the experiment (Fig. 2M). On the peak days, extracellular MC levels (DT2/MT2 and DT3/MT3) were significantly higher than those of the control in all cases except that of *Planktothrix* strain 1264 in the DT treatment (Table 1). For *Microcystis* strain 88 the peak extracellular MC value in the DT3 treatment was significantly higher than the peak value in the DT1 treatment; likewise for *Microcystis* strains 88 and

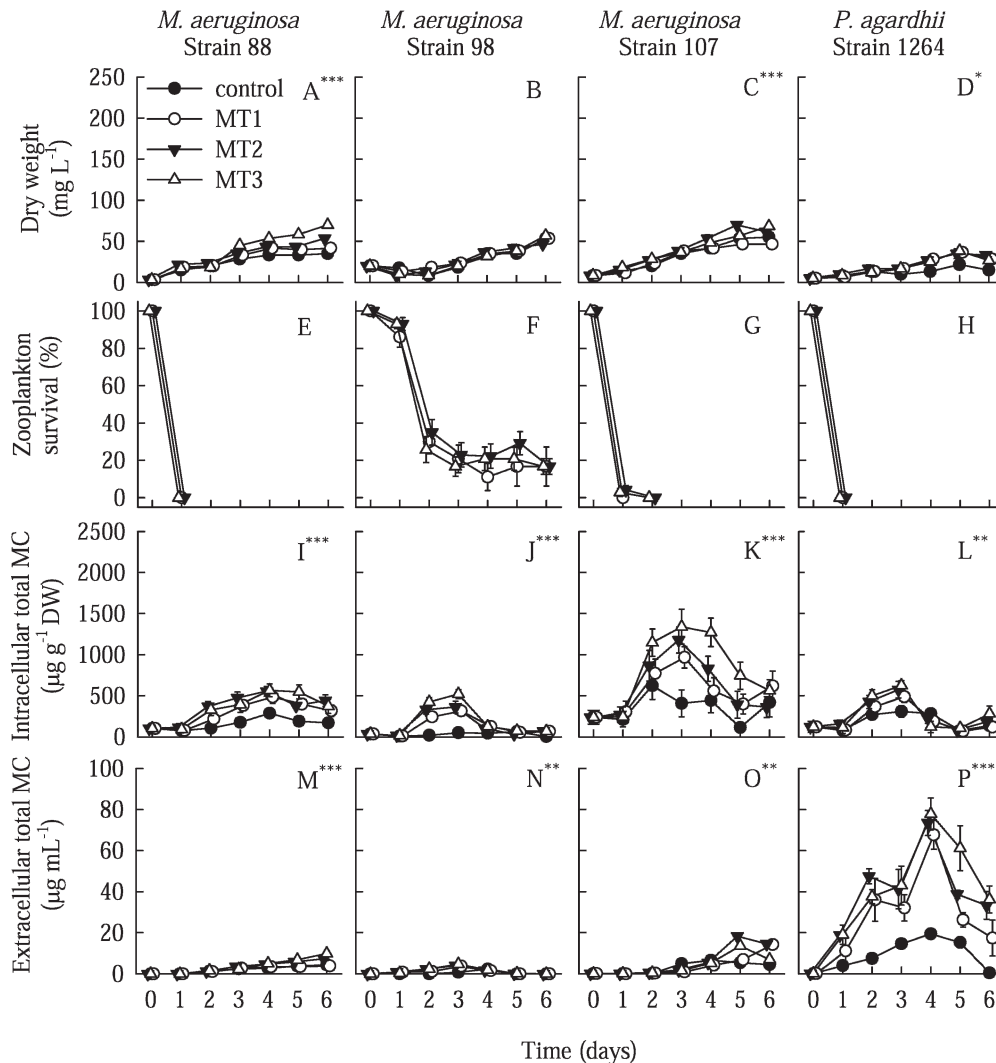


Fig. 2. Changes in cyanobacterial dry weight, zooplankton survival rate, intracellular MC, and extracellular MC when four cyanobacterial strains (three strains of *Microcystis aeruginosa* and one strain of *Planktothrix agardhii*) were directly exposed to *Moina macrocopa* at three population densities (two, four, or eight individuals) or to the control. Data are means \pm SE ($n = 3$). Significant differences between controls and treatments on the basis of RM-ANOVA test are indicated by $+p < 0.1$, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

107 the peak values were significantly higher in treatment MT3 than in MT1 (Table 1). *P. agardhii* (strain 1264 exposed to MT) produced greater levels of extracellular MC than did the three *M. aeruginosa* strains.

Indirect-exposure experiment—Although exposure to ZCMF stimulated the growth of all four cyanobacterial strains (Figs. 3A–D, 4A–D), the change in biomass did not differ significantly among the three ZCMF concentrations (RM-ANOVA and post hoc Tukey test, $p > 0.05$). The patterns of nutrient concentrations were similar to those of the direct-exposure experiments, showing a decrease in DIN (from 30–32 mg L⁻¹ on day 0 to 23–31 mg L⁻¹ on day 6) and an increase in DIP (from 0.9–2.5 mg L⁻¹ on day 0 to 1.9–4.9 mg L⁻¹ on day 6) over the course of the experiment.

For all four strains the intracellular MC levels were significantly different among the control and three treatments, and the intracellular MC levels increased after exposure to *Daphnia* and *Moina* ZCMF (Figs. 3E–H, 4E–H). Over the course of the experiment, the intracellular MC level was significantly higher in DCMF3/MCMF3 than in DCMF1/MCMF1 (RM-ANOVA and post hoc Tukey test, $p < 0.05$), except in the case of *Planktothrix* strain 1264. The intracellular MC levels of all cyanobacterial strains peaked at days 3–5 in all ZCMF treatments (Figs. 3, 4). On the peak day, the intracellular MC levels in treatments DCMF2/MCMF2 and DCMF3/MCMF3 were significantly higher than the control, except in the case of *Planktothrix* strain 1264 in treatment MCMF2 (Table 2). The peak intracellular MC levels for *Microcystis* strains 88, 98, and 107 in treatment DCMF3 were significantly higher than

Table 1. One-way ANOVA examining *Daphnia magna* and *Moina macrocopa* population density differences in the direct-exposure experiments in terms of cyanobacterial strain (*Microcystis* strains 88, 98, 107; *Planktothrix* strain 1264) and MC concentration (intracellular and extracellular) on the peak day. A post hoc Tukey test was used to test the difference between control (C) and treatments, where + $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

MC	Species	Strain	Source	df	MS	F	p	Post hoc comparison
Intracellular	<i>Daphnia</i>	88	Density	3	14,421.076	12.494	0.002	C<DT1*
			Error	8	11,558.807			C<DT2**
			Total	11				C<DT3**
		98	Density	3	91,217.883	13.948	0.002	C<DT1+
			Error	8	6,539.654			C<DT2**
			Total	11				C<DT3***
		107	Density	3	1,051,336.64	7.714	0.010	
			Error	8	136,282.52			C<DT2*
			Total	11				C<DT3**
		1264	Density	3	124,377.953	7.041	0.012	
			Error	8	17,665.995			C<DT2+
			Total	11				C<DT3**
	<i>Moina</i>	88	Density	3	50,027.063	3.707	0.061	
			Error	8	13,496.769			C<MT2+
			Total	11				C<MT3+
		98	Density	3	114,044.070	13.984	0.002	C<MT1*
			Error	8	8,155.087			C<MT2*
			Total	11				C<MT3***
		107	Density	3	495,329.241	5.799	0.021	
			Error	8	85,414.153			C<MT2*
Total			11		C<MT3*			
1264		Density	3	57,349.428	3.271	0.080		
		Error	8	17,531.016				
		Total	11				C<MT3+	
Extracellular	<i>Daphnia</i>	88	Density	3	101.750	39.594	<0.001	C<DT1**
			Error	8	2.570			C<DT2***
			Total	11				C<DT3***
		98	Density	3	15.287	13.395	0.002	C<DT1**
			Error	8	1.141			C<DT2**
			Total	11				C<DT3**
		107	Density	3	27.497	8.483	0.007	
			Error	8	3.241			C<DT2**
			Total	11				C<DT3*
		1264	Density	3	47.429	1.973	0.197	
			Error	8	23.854			
			Total	11				
	<i>Moina</i>	88	Density	3	3.362	21.149	<0.001	
			Error	8	0.159			C<MT2**
			Total	11				C<MT3***
		98	Density	3	9.276	19.022	0.001	C<MT1**
			Error	8	0.488			C<MT2**
			Total	11				C<MT3***
		107	Density	3	9.408	13.497	0.002	
			Error	8	0.697			C<MT2***
Total			11		C<MT3**			
1264		Density	3	503.892	19.463	<0.001	C<MT1**	
		Error	8	171.731			C<MT2***	
		Total	11				C<MT3***	

those in treatment DCMF1; likewise, the peak levels in *Microcystis* strains 88 and 107 and *Planktothrix* strain 1264 in treatment MCMF3 were significantly higher than those in MCMF1 (Table 2). Upon exposure to DCMF2/MCMF2 or DCMF3/MCMF3, *Microcystis* strain 107 showed a greater increase in intracellular MC production than did the other strains, and its intercellular MC levels were 1.5–3.0 times higher than those of the control (Figs. 3, 4).

Compared with the direct-exposure experiments (Figs. 1, 2), greater levels of extracellular MC were produced in the indirect-exposure experiments (Figs. 3, 4). The extracellular MC levels were significantly different among the control and three levels of ZCMF treatments, and the extracellular MC levels of *Microcystis* strains 88 and 98 and *Planktothrix* strain 1264 increased after indirect exposure to DCMF and MCMF (Figs. 3I–L, 4I–L). Only for *Microcystis* strain 98 was the extracellular MC level significantly

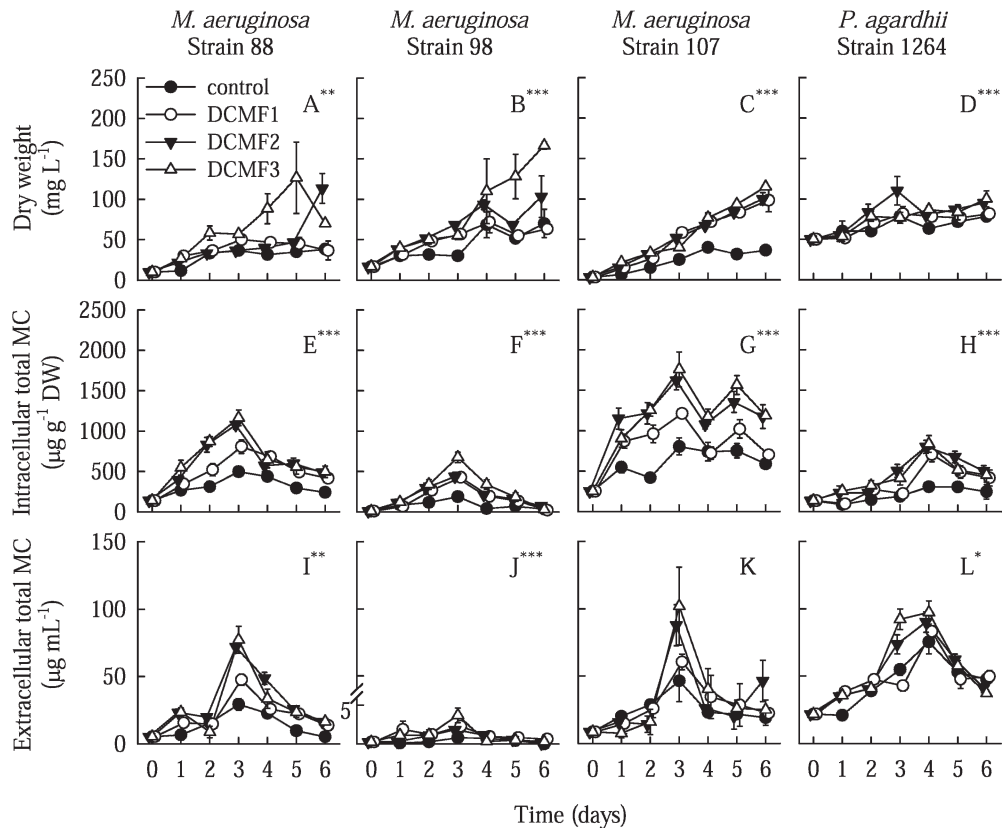


Fig. 3. Changes in cyanobacterial dry weight, intracellular MC, and extracellular MC when four cyanobacterial strains (three strains of *Microcystis aeruginosa* and one strain of *Planktothrix agardhii*) were exposed to three concentrations (0%, 25%, or 50%) of *Daphnia magna* culture media filtrate (indirect exposure) or the control. Data are means \pm SE ($n = 3$). Significant differences between controls and treatments on the basis of RM-ANOVA test are indicated by $^+p < 0.1$, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

higher in treatment MCMF3 than in MCMF1 (RM-ANOVA and post hoc Tukey test, $p < 0.05$). Similarly to the temporal patterns of intracellular MC level, the extracellular MC level peaked on day 3 or 4. The peak extracellular MC levels in treatments DCMF3/MCMF3 were significantly higher than those in DCMF1/MCMF1 for *Microcystis* strains 88 and 98 and *Planktothrix* strain 1264 (exposed to DCMF, MCMF, and MCMF, respectively; Table 2).

Correlation between MC production and zooplankton exposure—In the direct-exposure experiment only *Planktothrix* strain 1264 showed a statistically significant difference (RM-ANOVA, $p = 0.012$) in intracellular MC production between exposure to *D. magna* and *M. macrocopa*, whereas in the indirect-exposure experiment significant differences in intracellular MC production were observed in two cyanobacterial strains (*Microcystis* strain 88, RM-ANOVA, $p = 0.022$; *Planktothrix* strain 1264, $p = 0.010$). In the direct-exposure experiment three cyanobacterial strains (*Microcystis* strains 88 and 107 and *Planktothrix* strain 1264) showed statistically significant differences in extracellular MC between exposure to *D. magna* and *M. macrocopa* (RM-ANOVA, $p < 0.01$); in response to indirect exposure a significant difference in extracellular

MC between the DCMF and MCMF treatments was observed only in *Microcystis* strain 107 (RM-ANOVA, $p = 0.017$).

Assuming that the temporal patterns of intracellular MC production in the four cyanobacterial strains are similar upon exposure to either zooplankton species, a unimodal curve can be drawn. Examination of the unimodal curves for the patterns of intracellular MC concentration for all four strains in both zooplankton exposure experiments showed that the cyanobacteria showed peak MC production on days 3 to 4 (Fig. 5). In the direct-exposure experiments the maximum MC peak in the *Daphnia* treatments ($\sim 750 \mu\text{g g}^{-1}$ DW in DT3) was higher than that in the *Moina* treatments ($\sim 670 \mu\text{g g}^{-1}$ DW in MT3), whereas in the indirect-exposure experiments the maximum MC peak in the *Moina* treatments ($\sim 1,030 \mu\text{g g}^{-1}$ DW in MCMF3) was higher than that in the *Daphnia* treatments ($\sim 850 \mu\text{g g}^{-1}$ DW in DCMF3).

Overall, the levels of both intracellular and extracellular MC produced upon indirect exposure to *Daphnia* and *Moina* were higher than those upon direct exposure (Figs. 1, 2, 6). The slopes of log(extracellular MC) versus log(intracellular MC) upon direct exposure to *Moina* were not variable, irrespective of the increased levels of intracellular MC (Fig. 6B). In the indirect-exposure experi-

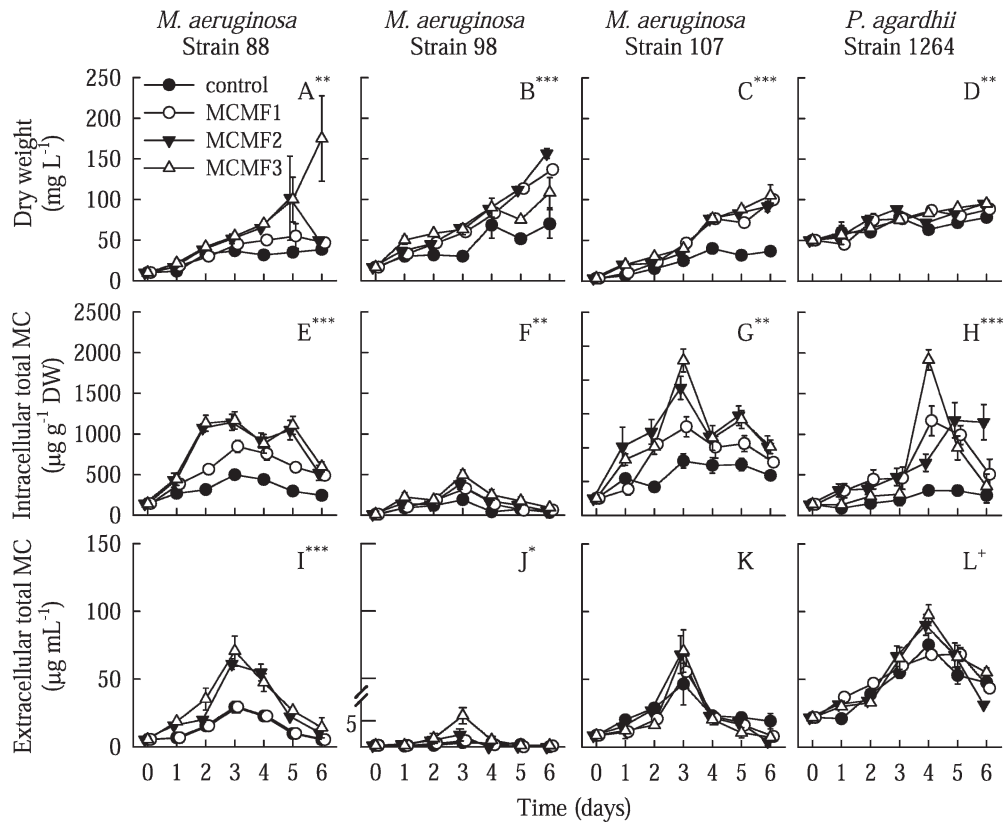


Fig. 4. Changes in cyanobacterial dry weight, intracellular MC, and extracellular MC when four cyanobacterial strains (three strains of *Microcystis aeruginosa* and one strain of *Planktothrix agardhii*) were exposed to three concentrations (0%, 25%, or 50%) of *Moina macrocopa* culture media filtrate (indirect exposure) or the control. Data are means \pm SE ($n = 3$). Significant differences between controls and treatments on the basis of RM-ANOVA test are indicated by $^+p < 0.1$, $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

ments the slopes of log(extracellular MC) versus log(intracellular MC) were similar in both zooplankton treatments (Fig. 6), whereas in the direct-exposure experiments the slopes were higher in the *Daphnia* treatments than in the *Moina* treatments.

Discussion

This study provides the first direct evidence of increased MC production by cyanobacteria in response to increasing zooplankton grazer densities and infochemical concentrations released by zooplankton. The significant correlation between exposure to zooplankton and MC content suggests that increased MC production serves as an inducible defense against zooplankton; this important finding is one of the first examples of induced chemical defenses in phytoplankton. The defense induced by both live animals and their exudates can be sufficiently explained by the presence of infochemicals, although the possibility of some direct effect of the presence of live animals cannot be completely excluded. These density- or concentration-dependent inducible defenses provide good support for our earlier study (Jang et al. 2003) in which we found differences in intracellular MC production caused by exposure to three zooplankton species (e.g., *D. magna*, *D.*

pulex, and *M. macrocopa*). However, that study focused on species-specific effects and did not reveal the effects of zooplankton density or dilution of the infochemicals. Our present results did not show species-specific effects, but they did reveal marked differences in both intracellular MC production and extracellular MC production by varying population densities and infochemical concentrations within the same zooplankton species. The density-dependent inducible defense revealed in the direct-exposure experiment provides valuable information, because the cyanobacteria responded to variations in zooplankton abundance at very realistic densities (i.e., 2–8 *D. magna* or *M. macrocopa* adults per 300 mL).

Examples of inducible defenses may be found in numerous organisms under selective pressure by predators, parasites, herbivores, pathogens, and even competitors (Tollrian and Harvell 1999). Tollrian and Harvell (1999) proposed four prerequisites for the classification of an inducible defense: (1) variable and sometimes strong selective pressure of an inducing agent; (2) a reliable cue to indicate threat and to activate defense; (3) effective defense; and (4) cost of defense (trade-off) must prevent the defense form being permanent. On the basis of our series of experiments, we can classify the production of MC by cyanobacteria as an effective induced chemical defense

Table 2. One-way ANOVA examining differences between *Daphnia magna* and *Moina macrocopa* zooplankton culture media filtrates in the indirect-exposure experiments in terms of cyanobacterial strain (*Microcystis* strains 88, 98, 107; *Planktothrix* strain 1264) and MC concentration (intracellular and extracellular) on the peak day. A post hoc Tukey test was used to test the difference between the control (C) and treatments (DF denotes *Daphnia* culture media filtrate; MF denotes *Moina* culture media filtrate), where + $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

MC	Species	Strain	Source	df	MS	F	p	Post hoc comparison	
Intracellular	<i>Daphnia</i>	88	Density	3	271,058.821	18.456	0.001	C<DF1+	DF1<DF2+
			Error	8	14,687.034			C<DF2**	DF1<DF3*
			Total	11				C<DF3***	
		98	Density	3	117,537.987	13.250	0.002	C<DF1+	
			Error	8	8,870.926			C<DF2+	DF1<DF3*
			Total	11				C<DF3***	DF2<DF3+
		107	Density	3	306,335.179	10.043	0.004		
			Error	8	30,502.577			C<DF2*	DF1<DF3+
			Total	11				C<DF3**	
		1264	Density	3	177,829.299	8.170	0.008	C<DF1*	
			Error	8	21,765.528			C<DF2*	
			Total	11				C<DF3**	
	<i>Moina</i>	88	Density	3	294,135.130	14.633	0.001	C<MF1+	
			Error	8	20,100.180			C<MF2**	MF1<MF3+
			Total	11				C<MF3**	
		98	Density	3	47,655.970	6.854	0.013		
			Error	8	5,685.957			C<MF2+	
			Total	11				C<MF3**	
		107	Density	3	779,726.914	14.641	0.001		
			Error	8	53,255.840			C<MF2*	MF1<MF3*
Total			11		C<MF3***				
1264		Density	3	1,468,739.87	30.748	<0.001	C<MF1**	MF1>MF2+	
		Error	8	47,767.29			C<MF3***	MF1<MF3*	
		Total	11				C<MF3***	MF2<MF3***	
Extracellular	<i>Daphnia</i>	88	Density	3	1,493.966	14.769	0.001		DF1<DF2+
			Error	8	101.154			C<DF2**	DF1<DF3*
			Total	11				C<DF3**	
		98	Density	3	4.413	3.614	0.065		
			Error	8	1.221				
			Total	11				C<DF3*	
		107	Density	3	1,910.093	1.906	0.207		
			Error	8	1,002.169				
			Total	11					
		1264	Density	3	263.247	1.549	0.276		
			Error	8	169.955				
			Total	11					
	<i>Moina</i>	88	Density	3	954.229	6.733	0.014		
			Error	8	141.725			C<MF2*	
			Total	11				C<MF3*	
		98	Density	3	16.911	5.229	0.027		
			Error	8	3.234				MF1<MF3*
			Total	11				C<MF3*	
107	Density	3	393.33	0.637	0.612				
	Error	8	617.188						
	Total	11							
1264	Density	3	541.899	3.446	0.072				
	Error	8	157.136				MF1<MF3+		
	Total	11							

against zooplankton grazing. The selective pressure of filter-feeding zooplankton (e.g., cladocerans) as an inducing agent is variable and unpredictable under environmental conditions, but zooplankton are often strong filter feeders that ingest many cells or colonies of phytoplankton at a time. Thus, these inducible defenses are likely evolved in the response to filter-feeding zooplankton constantly

present in eutrophic fresh waters. When the zooplankton feed on cyanobacteria, which are a poor food source, the potentially toxic cyanobacteria respond to the presence of zooplankton by MC production. Water that contains live zooplankton may contain infochemicals that are released by the zooplankton and cause cyanobacteria to activate defensive MC production. This increased MC concentra-

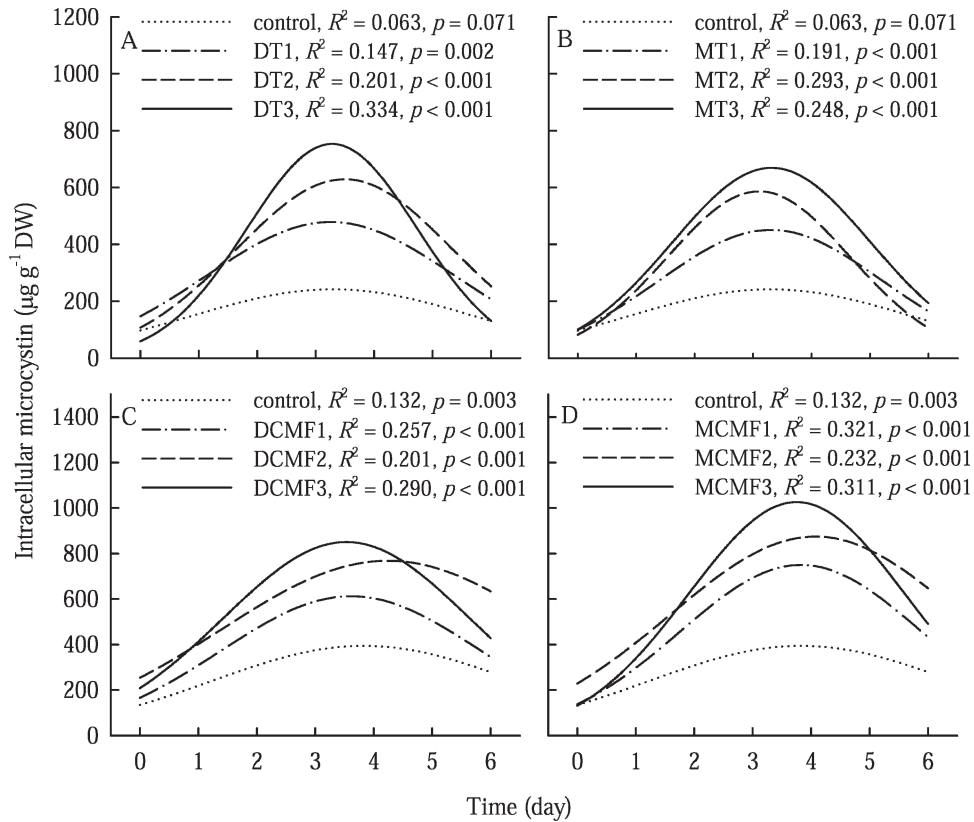


Fig. 5. Unimodal curve of intracellular MC concentration in both exposure experiments for both zooplankton species: (A) direct *Daphnia* exposure; (B) direct *Moina* exposure; (C) indirect *Daphnia* exposure; and (D) indirect *Moina* exposure.

tion may inhibit food intake by zooplankton and increase their mortality rates. Benefits of this induced cyanobacterial defense can be seen in the reduced zooplankton survivorships across the three zooplankton density treatments in this experiment. However, since there would be

high energy costs to the cyanobacteria in producing the defenses, the cyanobacteria should induce the defense mechanism only when necessary.

Several empirical studies of aquatic predator-prey interactions have reported that induced defenses are related

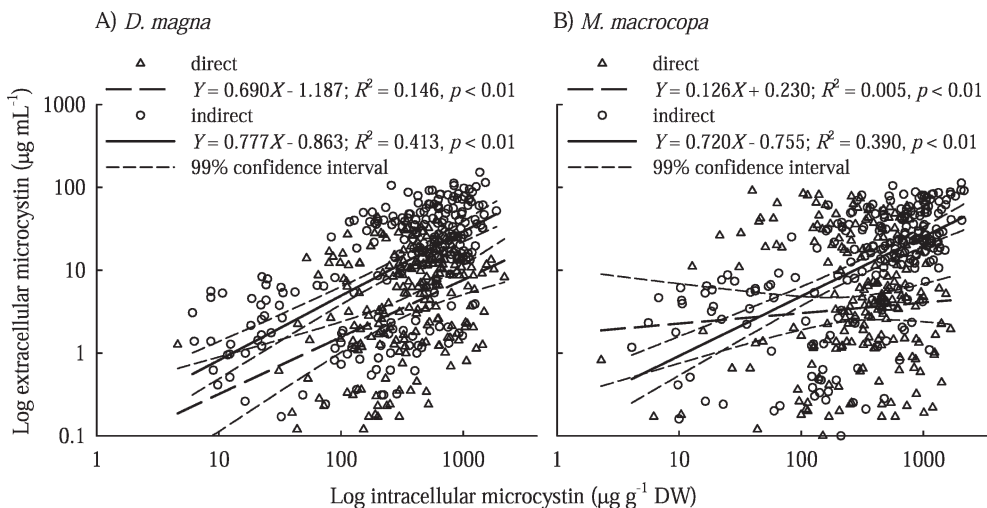


Fig. 6. Relation between log(extracellular MC) and log(intracellular MC) in all four cyanobacterial strains for both direct- and indirect-exposure experiments: (A) *Daphnia magna* treatments and (B) *Moina macrocopa* treatments.

to the intensity of consumer pressure. For example, Lampert et al. (1994) revealed that the induction of colony formation in *Scenedesmus* depends on the concentration of infochemicals released from *Daphnia* grazers. A study by Fiałkowska and Pajdak-Stós (1997) suggested that a high density of the ciliate grazer *Furgasonia blochmanni* triggers *Phorimidium* cyanobacteria to congregate into dense clumps surrounded by a mucilage layer. This phenomenon has been observed in ciliated protozoan interactions as well. For instance, the extent of induced defensive morphological transformation of the egg-shaped protozoan *Euplotes* (Ciliophora, Hypotrichia) is correlated with the density or kairomone concentrations of predatory protozoan ciliates (e.g., *Lembadion*, *Stylonychia*; Kuhlmann et al. 1999; Wiackowski and Starońska 1999). Previous studies have investigated induced antipredator morphological, developmental, or behavioral responses of prey organisms with regard to predator density. Our study provided the first evidence of MC production by toxic cyanobacteria as an antipredator defense dependent on both zooplankton population density and infochemical concentration.

Our findings revealed that the magnitudes and peak values of intracellular MC and extracellular MC were higher when cyanobacteria were indirectly exposed to zooplankton via ZCMF than when directly exposed to their predators. This difference may reflect differences in the amounts of infochemicals released directly from the zooplankton versus that within the ZCMF. Although we did not perform qualitative or quantitative analyses of infochemicals, our findings suggested that infochemicals in the ZCMFs from 200 non-egg-bearing adult *Daphnia* or 300 non-egg-bearing adult *Moina* more strongly affected the cyanobacteria than did infochemicals released by two to eight zooplankton individuals during the direct-exposure experiment.

On the basis of the temporal pattern of MC production in response to both exposure experiments, the peaks occurred on days 3 and 4, although the amount of toxins produced differed among the four cyanobacterial strains (Fig. 5). The dynamics of MC production showed a similar pattern when cyanobacteria were exposed to live zooplankton or to their isolated chemical exudates. The inoculated zooplankton died quickly in response to the algal toxins, and, as discussed by Jang et al. (2003), the chemical signal likely degrades over a timescale of days after direct exposure. This scenario seems likely in most cases in the present experiments as well, although in direct-exposure experiments using *Microcystis* strain 107 and *Planktothrix* strain 1264, the intracellular MC production tended to rise again slightly at the end of the experiments, perhaps representing a delayed response or the continued presence of infochemicals. Studies on the distribution of MC production and zooplankton under field conditions have shown that high levels of MC production are maintained over 3 months in response to naturally occurring freshwater zooplankton (Watanabe et al. 1992; Ferrão-Filho et al. 2002). Our series of experiments was conducted for only 6 d, so we were unable to evaluate whether the second rise in MC production would have been maintained in strains 107 and 1264.

Our direct-exposure experiments showed that most of the inoculated zooplankton in culture with cyanobacterial strains 88, 107, and 1264 were dead within 3 d, although zooplankton in culture with strain 98 showed a longer survival rate. Several causes for the adverse effects of cyanobacteria on zooplankton have been proposed (e.g., Lürling 2003). On the basis of our observations, the death of zooplankton might be attributed to increased MC content or high cyanobacterial density, or both. Over the course of this experiment, the cyanobacterial density was very high (10^3 to $>10^6$ cells mL⁻¹) because of the need to include enough matter for the MC analysis; this high density may have led to the high mortality rates observed. However, numerous studies have reported that many cyanobacteria possess toxins that might cause death in zooplankton, mainly *Daphnia* (DeMott et al. 1991). Considering that increased intracellular MC and extracellular MC reduced the survival rates of two filter-feeding cladocerans in this experiment, MC production does in fact seem to be an effective induced defense against zooplankton. Numerous laboratory studies have revealed that MC-producing strains of *M. aeruginosa* strongly inhibit feeding or food ingestion in zooplankton (e.g., Nizan et al. 1986), but evidence suggests that substances other than MC may also induce grazing inhibition in zooplankton. For instance, Rohrlack et al. (1999) showed that both an MC-free mutant and an MC-producing strain of *M. aeruginosa* reduce the filtration and ingestion of *Daphnia galeata*, whereas only the MC-producing strain has a lethal effect on the zooplankton. Our experimental design did not allow us to examine whether MC inhibited food ingestion in zooplankton, but future studies should investigate the potentially important role of other non-MC toxic compounds related to grazing inhibition.

The effect of grazer body size on induced defenses has been observed in studies of morphological changes in *Scenedesmus* (Lampert et al. 1994). In our direct-exposure experiment, the peak values of intracellular MC were higher in the *Daphnia* treatment (total dry weight, ~86 mg) than in *Moina* treatment (~27 mg). In addition, upon exposure to both zooplankton species, extracellular MC production by cyanobacteria increased with increasing intracellular MC production (Fig. 6). In the *Daphnia* treatment there was a higher slope of log(extracellular MC) versus log(intracellular MC) compared with the *Moina* treatments upon direct exposure (Fig. 6); this finding suggests that rates of MC excretion are correlated well with zooplankton metabolic and feeding rates, which are functions of body size.

Uncertainties remain regarding the mechanisms of extracellular MC release, although Rapala et al. (1997) reported that cell death or lysis releases intracellular toxins into the surrounding waters and the concentration of extracellular toxins increases as blooming toxic cyanobacteria age in eutrophic waters. In our study, however, extracellular MC increased with increasing zooplankton density in both the *Daphnia* and *Moina* treatments, similarly to the patterns of intracellular MC. In the direct-exposure experiments, extracellular toxins may have increased because of cell damage or degradation caused by

physical contact between the cyanobacteria and zooplankton, but extracellular MC also increased in the indirect-exposure experiments. Thus, our findings suggest that cyanobacteria release MC into the extracellular environment as an induced defense against zooplankton, and that this release is triggered by infochemicals produced by the zooplankton. A similar phenomenon was reported by Jang et al. (2006): when exposed to nontoxic cyanobacterial culture media filtrate for 6 d, toxic cyanobacteria released increasing levels of extracellular MC, a response that was also likely mediated by unknown chemicals from the allelopathic activity of the nontoxic cyanobacteria. 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 MCs can severely affect other organisms when released into the surrounding waters. Therefore, the ecological roles of extracellular MCs must be considered in studies of the biological interactions of toxic cyanobacteria and other food-web components in eutrophic fresh waters.

Recent studies on grazer resistance to toxic cyanobacteria (Hairston et al. 2001; Gustafsson et al. 2005) have shown that the tolerance of *Daphnia* to toxic *Microcystis* is an inducible defense developed during an individual's lifetime, and that this trait can be transferred to the next generation through genetic as well as maternal effects (Mousseau and Fox 1998). This transgenerational induction of inducible defenses may be an important component of predator-prey interactions (Agrawal et al. 1999). DeMott et al. (1991) reported that zooplankton have also evolved both physiological and behavioral adaptations that enhance their abilities to coexist with toxic cyanobacteria. Because *Daphnia* with this inducible resistance produce a great number of progeny, which show faster development to maturity and greater survival (DeMott et al. 1991), there are strong selective forces for this trait to evolve rapidly (Hairston et al. 2001). This inducible resistance to toxic cyanobacteria and inducible defenses against herbivorous zooplankton may explain the coexistence and coevolution of both organisms over time in eutrophic fresh waters. These genetic and coevolutionary aspects of inducible defenses remain to be clarified (Tollrian and Harvell 1999). Over the last decade, the biological control of toxic cyanobacterial blooms in eutrophic fresh waters has received increasing attention. Our observations of the mechanism underlying MC production in the presence of herbivorous zooplankton should help in devising an effective plan for the biological control of these organisms.

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