

Depuration Kinetics and Persistence of the Cyanobacterial Toxin Microcystin-LR in the Freshwater Bivalve *Unio douglasiae*

Atsushi Yokoyama, Ho-Dong Park

Department of Environmental Sciences, Faculty of Science, Shinshu University, Matsumoto 390-8621, Japan

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ABSTRACT: We carried out uptake and depuration experiments in the laboratory to investigate the effects of temperature (15°C and 25°C) on the depuration kinetics and persistence of a cyanobacterial toxin, microcystin-LR (MCYST-LR), in a freshwater bivalve, *Unio douglasiae*. Bivalves were fed toxic *Microcystis* cells in the 15-day uptake experiment and nontoxic diatoms in the following 15-day depuration experiment. Each bivalve's hepatopancreas was lyophilized and extracted with a butanol:methanol:water solution for analysis of MCYST-LR by high-performance liquid chromatography. The toxin in the organ accumulated rapidly after the beginning of the uptake experiment and reached approximately steady-state conditions on day 5 at concentrations of $130 \pm 11 \mu\text{g g}^{-1}$ dry weight at 15°C and $250 \pm 40 \mu\text{g g}^{-1}$ at 25°C. In the depuration experiments MCYST-LR was eliminated asymptotically from the tissue. The values of the depuration rate constant (k_d), calculated with a first-order one-compartment model, were found to be $0.142 \pm 0.044 \text{ day}^{-1}$ at 15°C and $0.226 \pm 0.046 \text{ day}^{-1}$ at 25°C. The depuration Q_{10} value from 15°C to 25°C equaled 1.6. This study was the first to reveal the kinetics of depuration for MCYST-LR in a bivalve. The results show that MCYST-LR may be eliminated slowly in autumn and winter and persist in the tissue until spring. Thus, in terms of toxicokinetics, the risk to people of being poisoned by bivalves would increase if toxic blooms occur in autumn. © 2003 Wiley Periodicals, Inc. *Environ Toxicol* 18: 61–67, 2003.

Keywords: microcystin-LR; toxicokinetics; accumulation; depuration rate constant; bivalve; *Unio douglasiae*

INTRODUCTION

Because of the eutrophication of waters, toxic cyanobacterial blooms occur in many countries, causing public health problems (Pitois et al., 2000) and damage to the aquatic ecosystem (Cristoffersen, 1996). One of the most common cyanobacterial toxins is microcystin (MCYST), produced by *Microcystis*, *Anabaena*, *Oscillatoria*, and *Nostoc* (Sivonen,

1996). MCYST is a cyclic heptapeptide including an unusual 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda; Botes et al., 1984). It is hepatotoxic in mammals (Yoshizawa et al., 1990; Solter et al., 1998). Sixty-five analogues of MCYST have been identified so far (Rinehart et al., 1994; Park et al., 2001). Among these, microcystin-LR (MCYST-LR) has been well studied and has been shown to have harmful influences on aquatic animals such as fish (Råbergh et al., 1991; Bury et al., 1995, 1997; Oberemm et al., 1997, 1999; Fischer et al., 2000) and zooplankton (DeMott et al., 1991). Some studies have reported adverse effects on fish populations in association

Correspondence to: Ho-Dong Park; e-mail: pparkhd@gipac.shinshu-u.ac.jp

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with toxic cyanobacterial blooms (Carbis et al., 1997; Ernst et al., 2001; Zimba et al., 2001).

Cyanobacterial toxins can also be accumulated by fish (Andersen et al., 1993; Sipilä et al., 2001a, 2001b; Zimba et al., 2001), bivalves (Eriksson et al., 1989; Falconer et al., 1992; Vasconcelos, 1995; Prepas et al., 1997; Watanabe et al., 1997; Williams et al., 1997; Amorim and Vasconcelos, 1999), gastropods (Zurawell et al., 1999), crayfish (Lirås et al., 1998; Saker and Eaglesham, 1999; Vasconcelos et al., 2001), and zooplankton (Watanabe et al., 1992; Kotak et al., 1996; Thstrup and Christoffersen, 1999). Because cyanobacterial hepatotoxins such as MCYST can promote tumors (Nishiwaki-Matsushima, 1992), contamination of seafood with the toxin poses serious public health risks and can damage the fishing industry (Vasconcelos, 1999; Van Buynder et al., 2001).

The persistence and detoxification of toxins in seafood are important issues for public health and fishery economics. Several investigations using different species of bivalves found differences in the depuration of MCYST (Eriksson et al., 1989; Vasconcelos, 1995; Prepas et al., 1997; Williams et al., 1997; Amorim and Vasconcelos, 1999). These differences may have resulted from the differences in species and experimental designs. Thus, the persistence of MCYST in bivalves remains unclear. Yokoyama and Park (2002) suggested that throughout winter MCYST persists in the freshwater bivalve *Unio douglasiae*, inhabiting the eutrophic natural Lake Suwa, where toxic *Microcystis* blooms have occurred during the summer since the 1970s (Park et al., 1993, 1998). Studies of toxicokinetics are required for accurate estimates of the accumulation, distribution, metabolism, and elimination of toxic compounds (Landrum et al., 1992). Although toxicokinetic studies of mammals have been done (Robinson et al., 1991; Stotts et al., 1997), thus far there have been no toxicokinetic studies involving contamination of aquatic organisms with cyanobacterial toxins. Therefore, the aims of this study were to reveal the depuration kinetics of MCYST and the persistence of the toxin in bivalves at different temperatures.

MATERIALS AND METHODS

Culture of Toxic *Microcystis*

For the uptake experiments we used a toxic strain of *Microcystis ichthyoblabe* (TAC95), producing the sole toxin, MCYST-LR, at a concentration of 13.7 mg g^{-1} of freeze-dried cells (Watanabe et al., 1991). The strain was cultured semicontinuously at 20°C in three glass carboys containing 10 L of the MA medium (Ichimura, 1979), with continuous aeration under white fluorescent lights ($40 \mu\text{E m}^{-2} \text{ s}^{-1}$) and with a 12-h light:12-h dark photoperiod. When the culture reached the early stationary phase, half the culture medium was harvested and centrifuged to collect toxic cells for the

uptake experiments. The carboy from which the old medium was removed was refilled with fresh medium. This harvesting process was repeated every 6 days.

Experimental Animals

Freshwater bivalves—*Unio douglasiae* (Unionidae)—were collected in June 1998 from the south littoral zone of Lake Suwa, central Honshu, Japan. Because these animals contained MCYST in their hepatopancreases at $>60 \mu\text{g g}^{-1}$ of dry weight (DW) (Yokoyama and Park, 2002), they were kept in a *Microcystis*-free pond in our laboratory for 3 months to eliminate the accumulated toxin completely. Sixty bivalves were then divided randomly into four groups of 15 bivalves and transferred from the pond into four glass aquaria containing 15 L of dechlorinated water. Each aquarium was aerated continuously. Clean artificial sea sand (Sea Sand B: 30–50 mesh; Nakalai tesque, Kyoto, Japan) was used as the bottom substrate. The bivalves were acclimatized to 15°C or 25°C for 10 days before the uptake experiments were begun. They were fed a maintenance diet of freeze-dried diatom, *Melosira varians* collected from a slow sand filter pond, at 10 mg DW L^{-1} every 2 days. The water was completely changed at the same time.

Uptake and Depuration Experiment

Uptake and depuration experiments were carried out at 15°C or 25°C in incubators. Each experiment used 30 bivalves with mean shell lengths of $70.0 \pm 2.96 \text{ mm}$ (15°C) and $67.0 \pm 4.60 \text{ mm}$ (25°C). During the 15-day uptake experiment, living toxic *Microcystis* cells were administered to the animals every 2 days at mean concentrations of $15.0 \pm 4.33 \text{ mg DW L}^{-1}$ (15°C) and $14.5 \pm 1.45 \text{ mg DW L}^{-1}$ (25°C). These concentrations of *Microcystis* can be found during blooms in Lake Suwa (Yokoyama and Park, 2002). The experimental water was aerated to supply oxygen for the bivalves and mix *Microcystis* cells and changed completely every 2 days. Three animals were collected on days 0, 1, 3, 5, 10, and 15 and dissected in order to obtain the hepatopancreases for MCYST analysis. The tissues were frozen at -30°C and lyophilized separately before extraction of the toxin.

The water in the tanks was completely replaced with freshly dechlorinated water on day 15, and the 15-day depuration experiment was conducted as above, except that the animals were fed freeze-dried nontoxic *Melosira* at 10 mg DW L^{-1} . Two or three animals were collected on days 16, 18, 20, 25, and 30 and treated as described above.

Toxin Analysis

To evaluate the concentration of MCYST administered to the bivalves, 200 mL of experimental water was filtered

through a glass microfiber filter (GF/C; Whatman, Maidstone, UK) and then freeze-dried. The *Microcystis* cells on the filter were extracted and analyzed with reverse-phase high-performance liquid chromatography (HPLC; Park et al., 1998).

Toxin in the hepatopancreas tissue was extracted using the following method (Yokoyama and Park, 2002). Briefly, 0.2 g DW of the tissue was stirred and extracted for 24 h in 10 mL of a butanol:methanol:water solution (5:20:75) at room temperature. After centrifugation (1 h, 18 000 rpm, 4°C), the supernatant was pooled at 4°C in the dark. The pellet was resuspended and reextracted twice with the same procedure. After the 3-day extraction was finished, the three pooled supernatants were combined and diluted with water to twice the volume. To remove the unwanted material, the sample was passed first through an ODS (octadecylsilane) silica gel cartridge (5 g; Chromatorex ODS, 100–200 mesh, packed into a polypropylene cartridge) and was then cleaned up with a silica gel cartridge (2 g; SepPak, Massachusetts, USA). The eluate from the silica gel cartridge with 70% methanol–water was evaporated to dryness, and then the residue was dissolved in methanol. The methanol solution was analyzed by HPLC.

The sample solution was applied to a reverse-phase HPLC system equipped with an ODS column (Cosmosil 5C18-AR, 4.6 × 150 mm; Nakalai, Japan). The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-9A pump coupled to an SPD-10A UV detector set at 238 nm, an SPD-M10A photodiode array detector, and a C-R6A integrator. The sample was separated with a mobile phase consisting of a methanol:0.05 M phosphate buffer (pH 3.0, 58:42) at a flow rate of 1 mL min⁻¹. The MCYST concentration was quantified by standard MCYST-LR provided by Dr. K.-I. Harada, Meijo University.

Kinetic Models

The depuration rate constant and biological half-life of MCYST were evaluated using a simple first-order one-compartment model (Spacie and Hamelink, 1982). Under steady-state conditions, the equation describing the kinetic uptake and depuration can be written as:

$$dC_f/dt = k_u C_w - k_d C_f \quad (1)$$

where k_u (mL g⁻¹ day⁻¹) is the uptake rate constant, k_d (day⁻¹) is the depuration rate constant, C_w (ng mL⁻¹) is the MCYST concentration in water, and C_f (ng g⁻¹ DW) is the MCYST concentration in tissue.

After the bivalves are transferred to clean water, the depuration rate constant k_d can be derived from the slope of the first-order depuration equation:

$$dC_f/dt = -k_d C_f \quad (2)$$

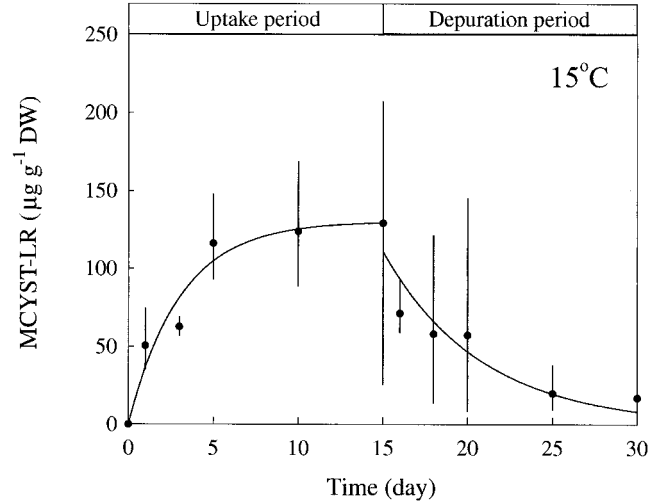


Fig. 1. Uptake and depuration of MCYST-LR in the hepatopancreas of *Unio douglasiae* ingested toxic *Microcystis* cells for the 15-day uptake experiment at 15°C. In the following 15-day depuration experiment (15°C), bivalves fed nontoxic diatom. Solid circles represent the average of three samples and vertical bars indicate maximum and minimum concentration of MCYST-LR in the tissue.

The integration of eq. (2) gives:

$$\ln C_f(t) = \ln C_f(0) - k_d t, \quad (3)$$

where $C_f(t)$ is the toxin concentration in the tissue at time t , $C_f(0)$ is the toxin concentration at the beginning of the depuration period, and t is time (day).

The biological half-life ($t_{1/2}$) of MCYST was computed from the equation:

$$t_{1/2} = \ln 2/k_d. \quad (4)$$

RESULTS

During the experiments only one bivalve died, on day 23 in the depuration experiment at 15°C. No adverse effects from the feeding behavior were found in the remainder of the bivalves. As a consequence of active feeding, the water always became clear, and aggregations of *Microcystis* cells, such as feces and pseudofeces excreted by the bivalves, were laid on the sand 1 day after administration of the cells.

The bivalves were exposed to MCYST-LR every 2 days at $27 \pm 4.27 \mu\text{g L}^{-1}$ (15°C) and $50 \pm 7.52 \mu\text{g L}^{-1}$ (25°C). The bivalves had eliminated all MCYST during the 3 months before the experiments began, because they contained no toxins on day 0 (Figs. 1 and 2). The animals rapidly accumulated MCYST-LR in their hepatopancreas on the day after the first exposure (Figs. 1 and 2). Approximately steady-state conditions during the uptake phase

were reached after 5 days at both temperatures: 130 ± 11 (15°C) and $250 \pm 40 \mu\text{g g}^{-1}$ DW (25°C). There was high variability in toxin concentrations on the same day. It is surprising that the bivalves could accumulate MCYST-LR to a maximum concentration of $630 \mu\text{g g}^{-1}$ DW (Fig. 2).

When the bivalves were transferred to clean water, MCYST-LR in the tissue declined asymptotically in the depuration experiments at both temperatures (Figs. 1 and 2). The values of the depuration rate constant (k_d) were $0.142 \pm 0.044 \text{ day}^{-1}$ at 15°C and $0.226 \pm 0.046 \text{ day}^{-1}$ at 25°C (Table I). The depuration Q_{10} value from 15°C to 25°C equaled 1.6. The depuration of MCYST-LR was more rapid at 25°C than at 15°C : the concentration was halved in 3.1 days at 25°C and in 4.9 days at 15°C . When the depuration experiments terminated on day 30, the bivalves still contained considerable amounts of the toxin in their hepatopancreases: $17.1 \mu\text{g g}^{-1}$ DW at 15°C and $8.4 \mu\text{g g}^{-1}$ DW at 25°C .

DISCUSSION

Toxicokinetic information is important for toxicological studies involved in the accumulation, distribution, metabolism, and elimination of toxic compounds (Landrum et al., 1992). This study is the first attempt to reveal the toxicokinetics of a cyanobacterial toxin, MCYST-LR, in a freshwater bivalve at different temperatures. We could not accurately estimate the uptake rate constant from our results because the exposure concentration of MCYST-LR [corresponding to C_w in eq. (1)] was not constant during the

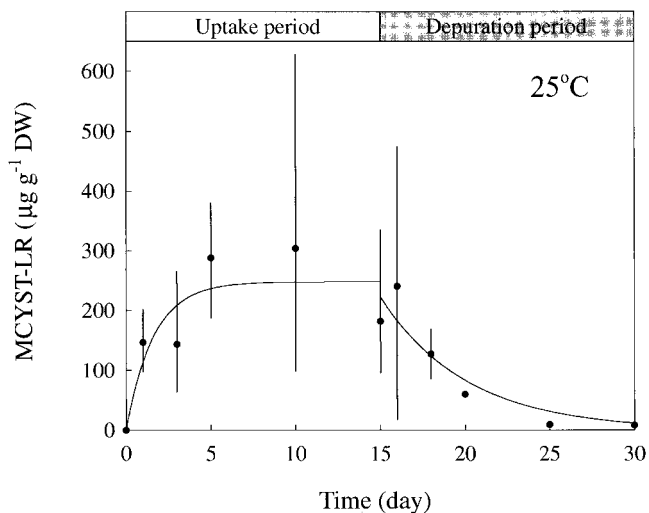


Fig. 2. Uptake and depuration of MCYST-LR in the hepatopancreas of *Unio douglasiae* ingested toxic *Microcystis* cells for the 15-day uptake experiment at 25°C . In the following 15-day depuration experiment (25°C), bivalves fed nontoxic diatom. Solid circles represent the average of two or three samples and vertical bars indicate maximum and minimum concentration of MCYST-LR in the tissue.

TABLE I. Kinetic coefficients for depuration experiments in first-order one-compartment model

	Temperature	
	15°C	25°C
k_d (day^{-1})	0.142 (0.044)*	0.226 (0.046)**
$t_{1/2}$ (day)	4.9	3.1
r^2	0.39*	0.67**

k_d is the depuration rate constant (standard error) and $t_{1/2}$ is the corresponding biological half-life; r^2 is the coefficient of determination of the regression analysis; * $p < 0.01$, ** $p < 0.001$.

uptake phase as a result of the 2-day exposure intervals, and thus the experiments did not fulfill the requirements for a kinetic model (Spacie and Hamelink, 1982). However, the depuration rate constants appear reliable as the MCYST-LR concentration in the tissue had already reached a steady-state condition by the time the depuration experiments started. MCYST-LR has a low tendency to bioconcentrate from water into aquatic organisms (Prepas et al., 1997): the log n-octanol/water distribution ratio of MCYST-LR has been found to be very low, ranging from 2.18 to -1.76 as pH increased (de Maagd et al., 1999). The depuration rate constant (k_d) of hydrophilic MCYST-LR in *U. douglasiae* of 0.226 day^{-1} at 25°C was higher than the values of 0.042 – 0.172 day^{-1} for hydrophobic polychlorinated biphenyl (PCB) congeners, whose log n-octanol/water partition coefficients (log K_{ow}) ranged from 5.6 to 7.5 in a study using zebra mussel (*Dreissena polymorpha*) by Morrison et al. (1995). In contrast, the k_d of MCYST of 0.142 day^{-1} at 15°C equaled those of PCB congeners with log $K_{ow} < 6.0$.

There is little information on the effects of temperature on the depuration rates of organic contaminants in bivalves. Fisher et al. (1993) found that temperature correlated negatively with the depuration rates of PCBs in zebra mussel. However, Gossiaux et al. (1996) found that depuration rates of PCBs and polycyclic aromatic hydrocarbons in zebra mussel did not change significantly with temperature. In the freshwater clam *Pisidium amunicum*, k_d for hydrophilic 2,4,5-trichlorophenol was reduced at low temperature (4°C), but there was no temperature dependency for the hydrophobic benzo[a]pyrene (Heinonen et al., 2000). In a study by Johnston et al. (1999) using the hepatocytes of fish, which are heterotherms as are bivalves, the excretion rate of benzo[a]pyrene metabolites (phase II) in sablefish (*Anoplopoma fimbria*) hepatocytes accelerated with temperature ($Q_{10} = 1.67$), but there was no temperature dependency in hepatocytes of two other species of fish. The primary factors determining these responses to changes in temperature may be the difference in species and the adaptations to different thermal environments (Johnston et al., 1999). In our study temperature affected the toxicokinetics of MCYST-LR in *U. douglasiae*.

A few studies have discussed the depuration mechanisms of MCYST in bivalves. The freshwater bivalve *Anodonta cygnea* (Unionidae) retained detectable *Oscillatoria* toxin for 2 months in clean water (Eriksson et al., 1989). Prepas et al. (1997) carried out a 21-day depuration experiment with MCYST using *A. grandis simpsoniana* and found that the depuration was biphasic and that very little toxin was eliminated between days 6 and 21. In the marine mussel *Mytilus galloprovincialis*, MCYST was eliminated with fluctuations (Vasconcelos, 1995; Amorim and Vasconcelos, 1999). This kind of depuration process may be a result of cycles in the production and degradation of a protein phosphatase–MCYST adduct (Vasconcelos et al., 2001). The activity of detoxification enzymes such as glutathione-S-transferase and glutathione peroxidase increased in embryos of zebra fish (*Danio rerio*) exposed to MCYST-LR (Wiegand et al., 1999). Pflugmacher et al. (1998) demonstrated that MCYST was detoxified as a result of conjugation with glutathione in various aquatic organisms, including zebra mussel, fish, and daphnid. The physiology and biochemistry of heterotherms may be greatly influenced by changes of temperature. In *U. douglasiae*, the depuration rate constant for MCYST-LR might decrease as a consequence of a decline in the production of detoxification enzymes and delay of cycles in the production and degradation of protein phosphatase–MCYST adduct at lower temperatures. In winter the water temperature falls to about 5°C in Lake Suwa. Consequently, very little MCYST will be eliminated by *U. douglasiae*; we found high concentrations of MCYST in the hepatopancreas despite low concentrations in the lake water in December (Yokoyama and Park, 2002). This is consistent with our results showing that the toxin concentration at 15°C was higher than that at 25°C when the depuration experiments terminated.

We administered the same quantity of toxic *Microcystis* to bivalves at both 15°C and 25°C, but the exposure concentration of MCYST-LR in the 25°C experiment was twice that in the 15°C experiment. This difference was a result of the use of *Microcystis* cells from a semicontinuous culture system, in which it was difficult to maintain constant conditions, unlike with a continuous-culture chemostat system. Watanabe et al. (1989) showed that the MCYST content of a toxic strain of *M. viridis* changed during growth in batch culture. The difference in the steady-state toxin concentration in the bivalves between the two temperatures resulted from the exposure to different concentrations during the uptake experiments. Large differences were found in the toxin concentrations of the bivalves on days 15 and 20 in the 15°C experiment and on days 10 and 15 in the 25°C experiment. These variations did not derive from toxin dilution by tissue growth because body weights of the bivalves did not change through the uptake and depuration experiments, which could be explained using the depuration kinetic model. Variations in same sampling date were probably caused by fluctuation in the feeding behavior.

The reason for the death of one bivalve in the 15°C depuration experiment is unknown. No adverse effects were observed in bivalves exposed to toxic cyanobacteria during uptake studies by Eriksson et al. (1989) with *Anodonta cygnea*, and by Vasconcelos (1995) with *Mytilus galloprovincialis*. Negri and Jones (1995) reported high mortality caused by high temperature in the freshwater bivalves *Alathyria condola* when exposed to the toxic cyanobacterium *Anabaena circinalis*, which produces paralytic shellfish poisoning toxins. The tolerance of bivalves to environmental stresses may decline as a result of exposure to toxic cyanobacteria.

Our study was the first to reveal the kinetics of the depuration of a cyanobacterial toxin, MCYST-LR, in a bivalve. Our results suggest that *Unio douglasiae* can eliminate the toxin more rapidly at higher temperatures in summer. Toxin concentrations in the tissue will closely track temporal fluctuation of the ambient toxin concentrations, as reported by Yokoyama and Park (2002). Because the depuration rate constant for MCYST declined with decreasing temperature, the toxin can persist in the tissue until spring. Thus, in terms of toxicokinetics, the risk to humans from eating bivalves will be increased if toxic blooms occur in autumn.

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