

Accumulation and elimination of cyanobacterial hepatotoxins by the freshwater clam *Anodonta grandis simpsoniana*

E.E. Prepas, B.G. Kotak, L.M. Campbell, J.C. Evans, S.E. Hruday, and C.F.B. Holmes

Abstract: Freshwater clams (*Anodonta grandis simpsoniana*) exposed to 51–55 $\mu\text{g}\cdot\text{L}^{-1}$ of dissolved microcystin-LR (MC-LR) in the laboratory for 3 days did not accumulate MC-LR equivalents (MC-LReq). However, clams placed in three eutrophic lakes with phytoplankton containing MC-LR (concentrations from below detection to 8.3 $\mu\text{g}\cdot\text{L}^{-1}$ cellular toxin) for 12–28 days accumulated the toxin (24 ± 7 to 527 ± 330 $\text{ng}\cdot\text{g}^{-1}$ MC-LReq; mean \pm SE). The relative MC-LReq concentrations in clams reflected MC-LR concentrations in lake phytoplankton, but individual variation was high. In individual clams exposed for 24 days, the average MC-LReq concentration was usually greater in the visceral mass than in gills and muscle, but average toxin concentrations in the three tissues were similar (587, 310, and 364 $\text{ng}\cdot\text{g}^{-1}$ dry weight⁻¹). In clams removed from the lake and placed in toxin-free water, MC-LReq concentrations in tissues declined rapidly for 6 days (by 69–88%) but remained relatively stable for the remaining 15 days. Analysis of clam tissues appears to be a more sensitive MC-LR indicator than analysis of phytoplankton. Accumulation of potent cyanobacterial toxins by this clam warrants further study as many are consumed by muskrats (*Ondatra zibethicus*), which in turn are consumed by terrestrial predators.

Résumé : Des anodontes du Nord (*Anodonta grandis simpsoniana*) exposés à 51–55 $\mu\text{g}\cdot\text{L}^{-1}$ de microcystine-LR (MC-LR) dissoute en laboratoire pendant 3 jours n'ont pas accumulé des équivalents de MC-LR (MC-LReq). Toutefois, des anodontes placés dans trois lacs eutrophes avec du phytoplancton contenant de la MC-LR (en-deçà du seuil de détection jusqu'à 8,3 $\mu\text{g}\cdot\text{L}^{-1}$ de toxine dans la cellule) pendant 12-28 jours ont accumulé la toxine (24 ± 7 à 527 ± 330 $\text{ng}\cdot\text{L}^{-1}$ de MC-LReq; moyenne \pm erreur témoins). Les concentrations relatives de MC-LReq dans les anodontes reflétaient les concentrations de MC-LR dans le phytoplancton du lac, mais la variation individuelle était élevée. Dans des anodontes individuels exposés pendant 24 jours, la concentration moyenne de MC-LReq était habituellement plus élevée dans les viscères que dans les branchies et les muscles, mais les concentrations moyennes de toxine dans ces trois tissus (587, 310 et 364 $\text{ng}\cdot\text{g}^{-1}$ de masse sèche) étaient semblables. Chez des anodontes retirés du lac et placés dans de l'eau exempte de toxine, les concentrations de MC-LReq dans ces trois tissus ont diminué rapidement pendant 6 jours (réduction de 69 à 88%), mais sont demeurées relativement stables pour les 15 jours restants. L'analyse de la toxine présente dans les tissus d'anodonte semble être un indicateur plus sensible de la présence de MC-LR que l'analyse du phytoplancton. L'accumulation d'hépatotoxines cyanobactériennes puissantes par ces anodontes justifie des études plus poussées, parce que bon nombre sont consommés par des rats musqués (*Ondatra zibethicus*) qui, à leur tour, sont consommés par des prédateurs terrestres.
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Introduction

Microcystins are cyclic peptides produced by freshwater cyanobacteria including species of *Microcystis* (Namikoshi et al. 1992; Kotak et al. 1993, 1995), *Anabaena* (Krishnamurthy et al. 1986; Harada et al. 1991; Sivonen et al. 1992), and *Oscillatoria* (Meriluoto et al. 1989; Bruno et al. 1992). Accidental ingestion of microcystins has been responsible for the

poisoning and death of livestock, wildlife, and pets worldwide (Schwimmer and Schwimmer 1968; Beasley et al. 1983; Edler et al. 1985) and has been implicated in human-related illness (Falconer 1989; Lawton and Codd 1991). More than 50 microcystins have been structurally characterized. Exposure of laboratory mice to microcystin-LR (MC-LR) by intraperitoneal injection causes massive hemorrhage in the liver and subsequent death within a few hours (Kotak et al. 1993). Laboratory mice exposed (for up to 1 year) to *Microcystis* bloom extracts at various concentrations in their drinking water suffered effects ranging from increasing incidence of respiratory infections to liver injury causing death (Falconer 1989). Similar liver damage was also reported in studies using sheep as the test organism (Falconer 1989). There is little information on the links between aquatic food chains and cyanobacterial toxins; this paper addresses the potential for aquatic organisms, such as clams, to accumulate microcystins and pass them on up the food chain.

At present, most of the toxicological studies in animals have focused on laboratory mammals (e.g., rats or mice). A small

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but growing body of literature is developing on the relationship between toxic cyanobacteria and other components of aquatic food chains, including zooplankton and fish. Many of these studies, however, have focused only on the acute toxicity of hepatotoxins such as MC-LR to zooplankton (Lampert 1982; Fulton and Paerl 1987; DeMott et al. 1991; Rothhaupt 1991) or fish (Phillips et al. 1985; Sugaya et al. 1990; Rabergh et al. 1991). Few studies have focused attention on possible accumulation of microcystins in aquatic food webs (Eriksson et al. 1989; Lindholm et al. 1989; Watanabe et al. 1992), and only one has attempted to relate in situ concentrations of the toxin in cyanobacteria with those in higher trophic levels (Kotak et al. 1996).

Our present study examines the accumulation and elimination of cyanobacterial hepatotoxins, expressed as MC-LR equivalents (MC-LReq), by the freshwater unionid clam *Anodonta grandis simpsoniana*. Unionid clams are sessile filter-feeders and, as such, have great potential to accumulate toxins and act as bioindicators of even low toxin concentrations. In addition, muskrats (*Ondatra zibethicus*) feed heavily on clams (Hanson et al. 1989) and may transfer cyanobacterial toxins to terrestrial food chains, while diving ducks of the genera *Melanitta* and *Aythya* (which feed extensively on marine clams in the winter; Terres 1980) may also be occasional consumers of small freshwater clams. Clams were transported from an oligo-mesotrophic lake to (i) laboratory aquaria containing dissolved MC-LR and (ii) three hypereutrophic lakes in central Alberta that had cyanobacteria containing MC-LR. In addition, we determined the relative importance of clam visceral mass, gills, and muscle tissues in the accumulation and depuration of MC-LReq.

Materials and methods

Accumulation of MC-LReq by clams

In the summer of 1992, clams (*Anodonta grandis simpsoniana*) were collected by divers from Narrow Lake, located 140 km north of Edmonton, Alberta. Clams selected for this study were 65–75 mm in length and had 5–10 annuli. They were transported to the laboratory in 20-L plastic buckets containing Narrow Lake water and then transferred to aquaria within the same day. The aquaria contained clean quartz sand and dechlorinated water and were maintained at room temperature (20°C). Clams were allowed to acclimatize for 2–4 days and fed daily with a green algal culture (*Chlorella* sp. and *Scenedesmus* sp.).

Laboratory studies

A short-term experiment was performed to determine the accumulation of dissolved MC-LR by the clams. Thirty clams were placed into each of six 10-L aquaria as described above. Half of the water in each tank was removed daily and replaced with 4.5 L of fresh water to avoid buildup of wastes; 0.5 L of green algal culture was added to provide food for the clams. Sufficient MC-LR was added daily to three treatment aquaria over the 3-day experimental period to maintain a concentration of 55 µg·L⁻¹; no MC-LR was added to the remaining three (reference) aquaria. Prior to each MC-LR addition, two 7-mL subsamples of water were taken from each aquarium daily for MC-LReq monitoring.

Three clams per day were removed at random from each aquarium and the whole soft tissues removed from the shells, blotted dry, and frozen at -40°C. The soft tissues were then freeze dried and weighed, and MC-LReq was subsequently extracted in 80% methanol (0.3 g dry weight tissue·mL⁻¹) in a manner similar to that for okadaic acid

(Luu et al. 1993). MC-LReq concentrations in clams and in the water phase were determined by protein phosphatase (PP) bioassay (Holmes 1991; Boland 1993), with ³²P-radiolabelled phosphorylase *a* (a physiological substrate) and PP-1c enzyme. The PP bioassay detects all microcystins, reported here as MC-LR equivalents, that are not irreversibly bound to clam tissues (Holmes 1991). As the bioassay does not measure the fractions of accumulated toxins that are irreversibly bound to clam tissues, it underestimates the total clam toxin burden. In this study, the detection limit of the PP bioassay in clam tissue was 2 ng MC-LReq·g⁻¹ (dry weight), with a detection limit in the water phase of 0.05 µg MC-LReq·L⁻¹. A *t* test (*P* < 0.05) was used to determine if there were any differences between clams from treatment and control tanks.

Field experiment

Clams were placed in three eutrophic to hypereutrophic lakes in central Alberta (all are municipal drinking water supplies) on 11 August (Coal Lake) and 13 August 1992 (Little Beaver and Driedmeat lakes). In the summer of 1992, the range of MC-LR concentrations in the three study lakes varied from below detection (<0.005 µg MC-LR·L⁻¹, on the basis of phytoplankton cellular MC-LR and chlorophyll *a* concentrations) in Coal Lake to 0.82–1.92 µg MC-LR·L⁻¹ in Little Beaver Lake and 1.32–8.2 µg MC-LR·L⁻¹ in Driedmeat Lake. Twelve clams were placed into each of nine small (11 × 80 × 40 cm) plastic trays containing clean sand substrate and covered with wire mesh. A tray was placed at each of three locations in each lake (at a depth of 1 m below the water surface) and anchored to the lake bottom. Three live clams were removed at random from each tray on every sampling date; dead clams (identified by their lack of response to physical stimulus) were also removed at this time and discarded. Live clams were put on ice, frozen (within 2 h), then freeze dried. Fewer live clams were collected from Little Beaver Lake than from Coal and Driedmeat lakes because of mortality and the disappearance of two trays between 17 and 25 August. Clam mortality was higher in Little Beaver Lake (69%) than in Coal and Driedmeat lakes (8 and 28%, respectively) and controlled the lengths of the field trials in all three lakes, which ranged from 12 (Little Beaver Lake) and 18 days (Driedmeat Lake) to 28 days (Coal Lake).

Phytoplankton were collected for toxin analysis with a conical 64-µm mesh net (29 cm i.d. by 90 cm long, fitted with a brass plankton bucket), which was hauled vertically through the water column from a depth of 1 (Little Beaver Lake) or 2 m (Coal and Driedmeat lakes) to the lake surface. Phytoplankton samples were collected from 7 July to 30 September 1992 in Little Beaver and Driedmeat lakes and from 2 July to 29 September 1992 in Coal Lake. Phytoplankton were freeze dried and MC-LR was extracted twice with 5% acetic acid and analyzed by high-performance liquid chromatography (HPLC; Kenefick et al. 1992), with a detection limit of approximately 1 µg MC-LR·g dry weight⁻¹ of phytoplankton. In situations where MC-LR is the predominant microcystin present, the simpler and less expensive HPLC analysis of MC-LR gives results comparable to those obtained by PP bioassay (Lam et al. 1995) and is suitable for the analysis of MC-LR in water or phytoplankton. Chlorophyll *a* concentrations were determined spectrophotometrically after extraction in 95% ethanol (Bergmann and Peters 1980) and used to estimate phytoplankton biomass. Chlorophyll *a* concentrations were converted to dry weight concentrations of phytoplankton on the assumption that chlorophyll *a* represents, on average, 1.5% of phytoplankton ash-free dry weight (American Public Health Association et al. 1989). This allowed expression of MC-LR as micrograms (cellular) MC-LR per litre lake water (Kotak et al. 1995). All results in the text are given as mean ± standard error.

Depuration of MC-LReq from clams

Persistence of MC-LReq in the tissues of clams isolated from the source of toxin was examined in September 1993. Thirty-nine clams were collected from Narrow Lake on 27 August 1993. Four clams

were divided into the three basic body parts (visceral mass (intestine and gonad), gills, and muscle tissues (foot and adductor muscles)) and frozen for later determination of MC-LReq background concentrations. The remaining 35 clams were fed the green algal culture and kept in a 40-L Coleman cooler containing aerated Narrow Lake water until they were placed in Driedmeat Lake (on 30 August) for the accumulation phase of the experiment. Twenty-four days later, 32 of the live clams (2 had died) were removed from the lake and returned to the laboratory.

Four clams, representing day 0 of the depuration experiment, were immediately divided into the three body parts (visceral mass, gill, and muscle tissues) and frozen for later MC-LReq determination. The remaining clams were held in three 10-L aquaria containing dechlorinated water. One half (5 L) of the water in each aquarium was removed every 3 days and replaced with 4.5 L fresh water and 0.5 L of the green algal culture. Four clams were removed at random from the aquaria every 3 days and treated in the same manner as the day-0 clams. The depuration phase of the experiment lasted 21 days.

Results and discussion

Accumulation of cyanobacterial hepatotoxins by clams

Laboratory studies

Water in the treatment aquaria contained $51\text{--}55\ \mu\text{g}\cdot\text{L}^{-1}$ toxin over the 3-day experiment, while concentrations in the control aquaria were below detection ($\leq 0.05\ \mu\text{g}\cdot\text{L}^{-1}$). All of the clams filtered actively, and there were no indications of any adverse reaction to MC-LR. Three clams treated with MC-LR and two control clams from day 3 were analyzed for toxin accumulation. Extracts from control and treated clams had PP bioassay inhibition activities equivalent to MC-LR tissue concentrations of 5 ± 1 and $8 \pm 1\ \text{ng}\cdot\text{g}^{-1}$, respectively. Because MC-LR accumulation was not significant and fell within the detection limit for the methodology ($2\ \text{ng}\cdot\text{g}^{-1}$), no further analyses were performed. We concluded that there was no accumulation of dissolved MC-LR by clams over the 3-day period.

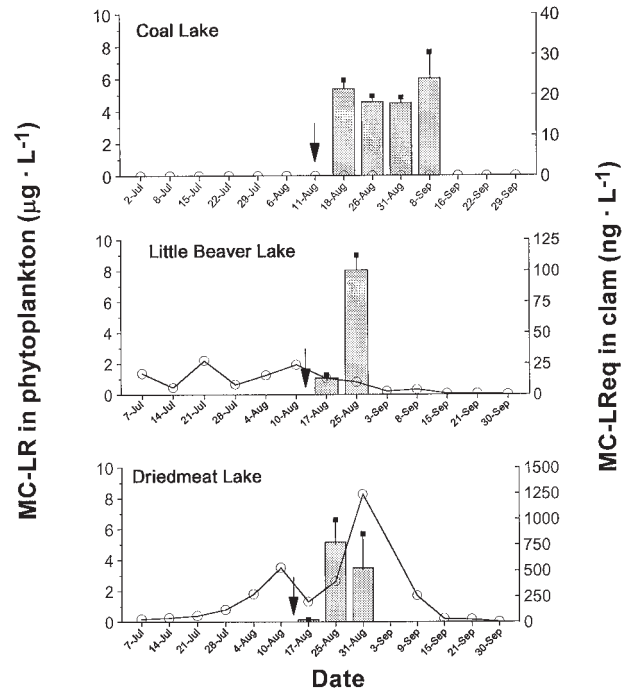
Field studies

The dominant cyanobacteria in the three lakes during the study were *Microcystis aeruginosa*, *Anabaena* spp., *Aphanizomenon flos-aquae*, *Phormidium* spp., and *Gomphosphaeria* sp. (Lam 1994). MC-LR was the major microcystin hepatotoxin analogue in these lakes (Craig et al. 1993). *Microcystis aeruginosa* was identified as the main MC-LR producer in the study lakes (Kotak et al. 1993, 1995).

The MC-LReq concentrations accumulated in clams varied from lake to lake. Clams that were exposed to phytoplankton containing relatively low proportions of *M. aeruginosa* ($\leq 3\%$ of the phytoplankton volume) with no detectable MC-LR concentrations (Fig. 1) in Coal Lake accumulated an average of $24 \pm 7\ \text{ng}\cdot\text{g}^{-1}$ over the 28-day experimental period. This accumulation shows that clams can bioconcentrate MC-LR to measurable concentrations in lakes where toxin concentrations in the phytoplankton are too low to be detected. The presence or absence of MC-LR in clam tissues may be a more sensitive indicator of MC-LR presence than that provided by direct measurement of phytoplankton toxin concentration.

Clams exposed to the phytoplankton of Little Beaver and Driedmeat lakes (Fig. 1) contained low levels of MC-LReq (13 ± 2 and $21 \pm 5\ \text{ng}\cdot\text{g}^{-1}$, respectively) after 4 days, which increased considerably (100 ± 12 and $776 \pm 215\ \text{ng}\cdot\text{g}^{-1}$,

Fig. 1. Microcystin-LR (MC-LR) concentrations in the phytoplankton (by HPLC; open circles) and microcystin (MC-LReq) concentrations accumulated in clams (whole clam, by PP bioassay; shaded bars) in Coal, Little Beaver, and Driedmeat lakes. Arrows indicate the dates when clams were placed into lakes. Note scale change in MC-LReq concentration across lakes. Error bars = 1 SE.

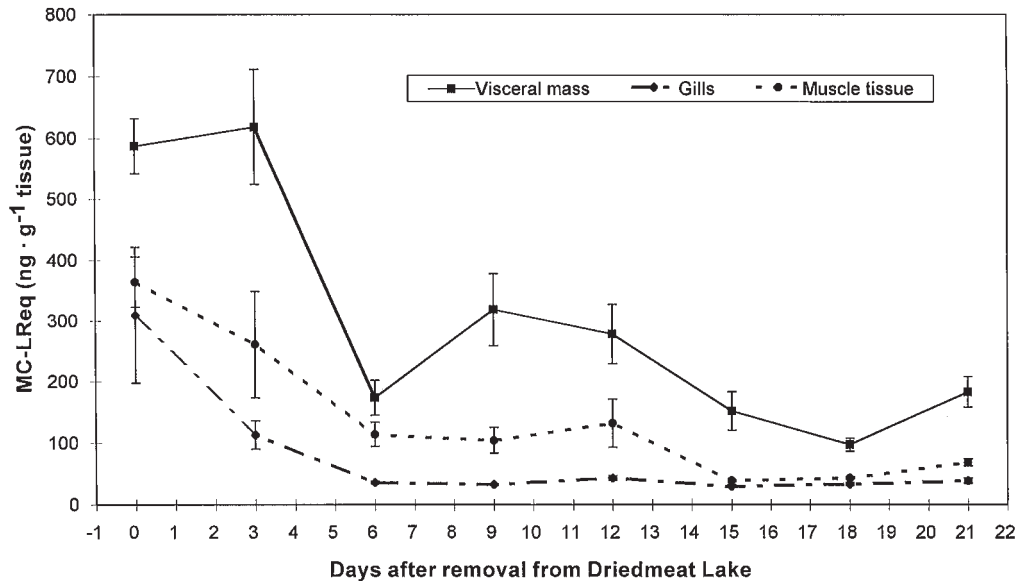


respectively) after 12 days of exposure. At the time of this experiment, the average MC-LR concentration in the phytoplankton of Little Beaver Lake was about half of that in Driedmeat Lake (1.26 ± 0.33 and $3.94 \pm 1.35\ \mu\text{g}\cdot\text{L}^{-1}$ lake water, respectively). In general, higher MC-LR concentrations in lake phytoplankton corresponded to the higher MC-LReq concentrations in clams.

The MC-LReq concentrations detected in clams also varied with site in each lake, reflecting the variability in both the spatial abundance of toxic *M. aeruginosa* (Kotak et al. 1995) and the filtering rates of the clams. For example, the ranges of MC-LReq concentrations in clams sampled from the three trays in Driedmeat Lake on 25 August were 1280–1350, 28–1210, and 30–610 $\text{ng}\cdot\text{g}^{-1}$ tissue. Clams sampled from Driedmeat Lake on 25 August contained the highest average MC-LReq concentration ($776 \pm 569\ \text{ng}\cdot\text{g}^{-1}$ tissue as compared with $527 \pm 739\ \text{ng}\cdot\text{g}^{-1}$ tissue on 31 August) found for this study.

The combined results of our laboratory and field studies suggest that clams accumulate MC-LReq primarily by grazing on toxic phytoplankton and minimally via uptake of the dissolved toxin. Clams exposed to relatively high ($55\ \mu\text{g}\cdot\text{L}^{-1}$) concentrations of dissolved MC-LR for 3 days did not accumulate significant levels of toxin, while those exposed to the far lower toxin concentrations present in lake phytoplankton ($<4\ \mu\text{g}\cdot\text{L}^{-1}$) accumulated measurable levels of MC-LReq (13 ± 2 and $21 \pm 5\ \mu\text{g}\cdot\text{L}^{-1}$, respectively, in Little Beaver and Driedmeat lakes) after only 4 days of exposure. These results are consistent with other studies that report that blue mussels

Fig. 2. Summary of the depuration study. Total microcystin (MC-LReq) concentrations in visceral mass, gills, and muscle tissue (foot and adductor muscles) of clams after exposure to toxic phytoplankton in Driedmeat Lake for 24 days (day 0) and subsequent confinement in laboratory aquaria with dechlorinated water and green algae for up to 21 days. Tissue concentrations are on a dry weight basis. Error bars = 1 SE.



(*Mytilus edulis*) accumulated ≥ 5 times more saxitoxin (Andrasi 1985) or domoic acid (Novacek et al. 1991) when the toxin was in phytoplankton rather than in solution.

Depuration of cyanobacterial hepatotoxins from clams

The background MC-LReq concentration for clams from Narrow Lake of 1993 was $6 \pm 1 \text{ ng} \cdot \text{g}^{-1}$ and the total toxin burden was divided evenly among the three body parts (26 ± 1 , 39 ± 5 , and $35 \pm 5\%$ in visceral mass, gill, and muscle tissues, respectively). In contrast, clams that were placed in Driedmeat Lake for 24 days during September 1993 (average MC-LR in phytoplankton was $2.78 \pm 0.61 \mu\text{g} \cdot \text{L}^{-1}$) accumulated an average MC-LReq concentration of $369 \pm 47 \text{ ng} \cdot \text{g}^{-1}$, and in general, more of the total toxin burden was present in the visceral mass ($55 \pm 10\%$) than in the gill or muscle tissues (23 ± 8 and $23 \pm 3\%$, respectively). The average MC-LReq concentrations in the visceral mass tissues of some individual clams after 24 days in Driedmeat Lake were far greater than in the gills and muscle; however, average toxin concentrations in the three tissues (587 ± 90 versus 310 ± 194 and $364 \pm 83 \text{ ng} \cdot \text{g}^{-1}$, respectively) were not different ($P > 0.05$) from each other.

Although it is possible that the high concentrations of MC-LReq in the visceral mass tissues of some clams were due to the presence of undigested toxic phytoplankton (as well as the MC-LReq that had penetrated into tissue intracellular spaces), this hypothesis is not supported by the patterns of toxin depuration in the three body parts (Fig. 2). When clams were removed from the toxin source and fed nontoxic green algae, 71% of the total accumulated MC-LReq was eliminated in the first 6 days, with 70, 88, and 69% loss in visceral mass, gill, and muscle tissues, respectively. The similar patterns of toxin loss in the three body parts suggest that the high concentrations of toxin found in the visceral mass tissues of some individual clams are due not to the presence of undigested toxic phytoplankton, but to increased exposure to, and possibly selective

bioconcentration of, MC-LReq in this body region. In another study on cyanobacteria, the amount of *Oscillatoria* toxin (an unidentified microcystin analogue) detected in the hepatopancreas of mussels (*Anodonta cygnea*) was 40% of the total toxin content, and toxin concentrations in this organ were more than eight times the concentrations found in other parts of the clam body (Eriksson et al. 1989).

The elimination of MC-LReq from the clams appeared to be biphasic. No further toxin losses were observed in the visceral mass and gill tissues between days 6 and 21 of the depuration experiment, while losses from muscle tissue increased slightly from 69% at day 6 to 81% at day 21. Overall, the 29% of the original free toxin that remained in the clams after 6 days was still present after 21 days, indicating that MC-LReq was present in both transitory and stable forms. These toxin compartments are in addition to the unknown amount of toxin that is irreversibly bound to clam tissues (and therefore not detectable by PP bioassay). Similar studies in the marine environment have shown that the phytoplankton toxins responsible for paralytic shellfish poisoning can remain in Alaska butter clams (*Saxidomus giganteus*) for more than a year (Shantz and Magnusson 1964).

The cyanobacterial toxins present in clams could also affect terrestrial food webs. Muskrats feed heavily on clams (Hanson et al. 1989); individuals consuming clams from Coal, Little Beaver, or Driedmeat lakes would clearly be exposed to MC-LReq and might subsequently pass on the toxins to their respective predators. This is of special concern as muskrats tend to select the larger (generally older) clams (Hanson et al. 1989), which are likely to have had the longest exposure to cyanobacterial toxins.

At present, studies relating to the impact of cyanobacterial hepatotoxins on aquatic food webs have concentrated primarily on the acute toxicity of microcystins on individual laboratory organisms such as mussels (Eriksson et al. 1989), zooplankton (Peñaloza et al. 1990; DeMott et al. 1991), and

fishes (Phillips et al. 1985; Peñaloza et al. 1990; Rabergh et al. 1991). Only two in situ studies have examined the toxin concentration in phytoplankton and zooplankton simultaneously (Watanabe et al. 1992; Kotak et al. 1996). Ours is the first study showing that (i) clams can be indicators of low levels of MC-LReq in phytoplankton in lakes and (ii) high toxin concentrations in phytoplankton could be linked to those in clams. In addition, our study showed that MC-LReq can persist in clams for ≥ 21 days with possible consequences for both aquatic and terrestrial food webs. Our study indicates the need for further examination of the accumulation of MC-LReq at different trophic levels.

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