Variation in the Response of Juvenile and Adult Gastropods (*Lymnaea stagnalis*) to Cyanobacterial Toxin (Microcystin-LR)

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ABSTRACT: Owing to the increasing public health problem related to the proliferation of toxin-producing cyanobacteria in aquatic ecosystems, we have investigated the response of the pond snail *Lymnaea stagnalis* exposed to 33 μg/L microcystin-LR for 6 weeks, through its life traits (survival, growth, fecundity) and locomotion; uptake of microcystin-LR was also quantified in the snail body tissues. To study the potential plasticity of the response related to the development stage, snails were exposed to the toxin as sexually immature and mature. According to our results, microcystin-LR accumulated in snail tissues at a higher level in juveniles (7.96 ng/g fresh weight) *versus* adults (2.17 ng/g fresh weight). Whatever the age, survival, growth, and locomotion were not affected by the toxin, and fecundity of polluted adults was reduced by half. These results are discussed in terms of negative effects of aqueous microcystin-LR occurrence on the dynamics of natural populations of gastropods. © 2005 Wiley Periodicals, Inc. Environ Toxicol 20: 592–596, 2005.

Keywords: *Lymnaea stagnalis*; microcystin; cyanobacteria; gastropod; life-traits; locomotion; bioaccumulation

INTRODUCTION

Natural populations of freshwater gastropods are subjected to severe ecological constraints imposed by large temporal fluctuations of their environment; their success depends on their physiological capacity to tolerate these fluctuations (Russel-Hunter, 1961). Blooms of cyanobacteria, especially in eutrophic waters during summer in temperate regions (Lindholm et al., 1989), can result after the collapse of the bloom and consequent cell lysis, in a sudden increase of dissolved toxins (microcystins) in the water column. Because of their toxicity for various organisms including humans (for reviews, Carmichael and Falconer, 1993; Christoffersen, 1996), microcystins constitute a natural health hazard in the environment and a growing problem in Europe (Skulberg et al., 1984), including Brittany (Vézie et al., 1996). Up to now, the cyanobacterial toxicity has not been considered to explain unusual fluctuations of natural gastropod populations. However, Zurawell et al. (1999) assessed the influence of lake trophic status on the occurrence of microcystin in the tissue of some pulmonate snails (*Lymnaea stagnalis, Physa gyrina, Helisoma trivolvis*), and Gevrey et al. (1972) demonstrated the mortality of *Radix auricularia* when exposed to biotoxins of *Microcystis farlowania* and *Pseudanabaena franquetii*, but no mortality after ingestion of these cyanobacteria.

The present study was undertaken to assess the impact of one of the most commonly occurring microcystins, the highly hepatotoxic microcystin-LR (MC-LR), on the life traits (survival, growth, fecundity) and the locomotory activity of the freshwater pulmonate *Lymnaea stagnalis*, and to quantify the possible accumulation of cyanotoxins in the snail tissues from dissolved microcystin. If *L. stagnalis* is unable to degrade MC-LR or if MC-LR is metabolized at a slower rate than it is ingested, the toxin will probably accumulate in the snails. Moreover, according to the

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TABLE I. Mean values ± standard error of fecundity (number of eggs and egg masses/snail/week) in *Lymnaea stagnalis* and snails exposed to 33 μg/L MC-LR, and of locomotion (distance covered in cm/10 min) and MC-LR accumulation (ng/g fresh weight) after 6 weeks of MC-LR exposure

	Eggs	Egg Masses	Locomotion	MC-LR Accumulation
Control juveniles	0	0	5.24 ± 1.07	0
Control adults	10.16 ± 1.80	0.24 ± 0.04	3.50 ± 1.35	0
Exposed juveniles	0	0	7.03 ± 0.78	7.96 ± 0.77
Exposed adults	5.34 ± 1.34	0.11 ± 0.03	1.69 ± 0.40	2.17 ± 0.43

different allocation patterns between individuals of the same species at different stages of their development (Boggs, 1992), we have compared the response of juveniles (sexually immature) and adults (sexually mature) when exposed to MC-LR. The response of the snails is expected to be different according to their age, related to the energy allocation pattern and immature *versus* mature immune system.

MATERIALS AND METHODS

The INRA Experimental Unit of Aquatic Ecology and Ecotoxicology (Rennes, France) provided us with *L. stagnalis* for this study. Snails are mass-reared under laboratory conditions (14/10 L/D, 20 ± 1 °C) in 30 L aquaria containing dechlorinated tap water and fed daily with pesticide-free lettuce. Prior to MC-LR exposure, 40 juvenile and 40 adult snails (13 ± 1 and 25 ± 1 mm shell length, respectively) were isolated in 75 mL glass containers, maintained at 20 ± 1 °C and 12/12 L/D, fed on dried lettuce leaves *ad libitum*, and acclimated to such conditions for 7 days. After acclimation, 20 juveniles and 20 adults were individually exposed for 6 weeks to 33 μg/L MC-LR (Alexis Corporation, San Diego, CA, USA), concentration in agreement with that in superficial fresh waters of Brittany during cyanobacterial blooms (up to 100 μg/L) (Brient et al., 2001). During the whole study, contaminated water was renewed every 7 days. Methanol (1 mL/L) was used to solubilize MC-LR. Controls (20 juveniles and 20 adults) were maintained in water with 1 mL/L methanol, renewed every week. Every week, the size of each snail was measured to the nearest 0.1 mm, and the egg masses laid were collected to record their number and the number of eggs per mass. After 6 weeks, locomotory activity was estimated from the distance moved by the snails over an allowed time. Each snail was placed for 10 min in a box filled with 75 mL water. After this period and following the removal of the snails, 10 mg of carmine was added to each box to adhere to the mucus tracks (Calow, 1974). The length of the mucus trails produced, revealed as red bands, was then measured with the help of a digital curvimeter to the nearest 1 mm (distance moved, *D*, measured in centimeters per 10 min).

For the measurements of bioaccumulation, MC-LR was extracted with 2 mL of 100% methanol (MeOH) (Codd

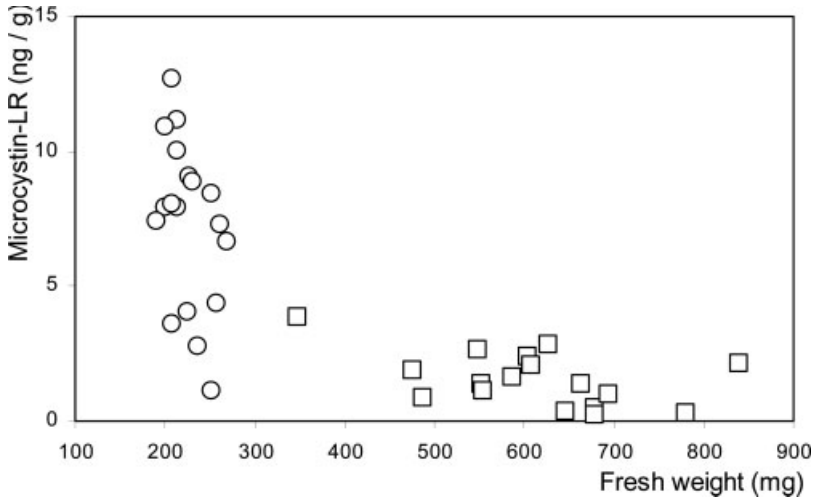

Fig. 1. Significant relationship between fresh body weight (*W*) and MC-LR concentration (*C*) in the tissue of *Lymnaea stagnalis* exposed as juvenile (circle) and adult (square) to 33 μg/L MC-LR. $C = 77927 \times W^{-1.73}$ (Spearman's coefficient = 0.81, $P = 0.0001$, $N = 35$).

TABLE II. Statistical comparisons (*t*-test with *T*, *P*, and DDL values) of locomotion between control *Lymnaea stagnalis* and snails exposed to 33 μg/L MC-LR, and of MC-LR accumulation between juveniles and adults

	T	*P*	DDL
Locomotion of control/ exposed juveniles	−1.36	−0.1819	38
Locomotion of control/ exposed adults	0.866	0.3922	36
Locomotion of control juveniles/adults	2.544	0.0158*	37
Locomotion of exposed juveniles/adults	6.006	0.0001*	37
Bioaccumulation of juveniles/adults	7.31	0.0001*	33

For the snails exposed to MC-LR, a significant relation was demonstrated between shell size (S) and distance moved (D). $D = -18.95$ Ln(S) + 63.31 (Spearman's coefficient = 0.59, $P = 0.0003$, $N = 39$).

*Significant at $P < 0.05$.

et al., 1997; Freita de Malgalhaes et al., 2001) from the body of each exposed snail (40 juveniles and 40 adults without shell). Analysis was made, after freezing of the tissues at −80°C in eppendorf tubes by immuno-assays, with ELISA microcystin Plate Kit (Envirologix, Portland, ME, USA): each snail was weighed, crushed in 1 mL of 100% MeOH, then crushed again after 12 h at 4°C (with 1 mL MeOH added), and centrifuged. The extract was diluted with water in order to obtain 10% MeOH for the analysis by immuno-assay (Codd et al., 1997). Analysis was carried out in triplicate for two dilutions: not diluted and half diluted. The method was able to detect microcystin from a level of 0.05 μg/L and to the nearest 0.01 μg/L.

Two-way analyses of variance (ANOVA) with repeated measure were performed, to compare the life-history parameters of control and exposed snails during the 6-week study. Multiple comparison tests (Scheffé and Fisher's PLSD) were performed when there was a significant interaction between time and microcystin exposure. Spearman's coefficient was calculated to assess the existence of a correlation between shell height and locomotion, and between shell height and MC-LR concentration in the tissues. The Student's t test was used for statistical comparisons of locomotion and MC-LR bioaccumulation between the different groups. Differences were considered to be statistically significant at $P < 0.05$. Data are reported as means ± standard error.

RESULTS AND DISCUSSION

Microcystins released after cyanobacterial lysis persist in water during 2 or 3 weeks; their concentration varies depending on environmental conditions and seasons, from 0 to 140 μg/L (Lindholm et al., 1989; Zurawell et al., 1999; Hyenstrand et al., 2003), and up to 1300–1800 μg/L following algicide treatment (Jones and Orr, 1994). According to the oral water ingestion rate of *L. stagnalis*, which ranged from 8 to 12 μL/h/g (De With, 1996), the uptake of MC-LR by the snails in our experiments takes place probably by direct ingestion. Transtegumental absorption of microcystin has never been shown, even if the uptake by the skin of particulate matter from the external medium has been found to occur in *L. stagnalis* (Zylstra, 1972).

Our findings assess that a direct exposure to aqueous MC-LR (33 μg/L) induces a moderate accumulation of the toxin within *L. stagnalis* tissues (Table I, Fig. 1): on average, 42 and 11 ng/g dry weight in juveniles and adults, respectively (according to the water percentage in fresh tissues, dry weight = 0.19 × fresh weight). Zurawell et al. (1999) demonstrated a greatly higher accumulation in *L. stagnalis* collected from lakes of varying trophic status (from 0 to 140 μg/g dry weight), and that the concentrations of MC-LR in snail tissues were correlated with toxin in the phytoplankton (from 0 to 1526 μg/g dry weight) and the relative abundance of *Microcystis* spp., but not with extracellular aqueous microcystin (from 0 to 1.3 μg/L). Besides, we show that MC-LR accumulation per body weight is significantly greater (about 3.7 times superior) in juveniles *versus* adults (Tables I and II), probably in relation to the development and the efficiency of immune system. The major defense mechanism of freshwater snails, in particular, in response to chemical and biological stressors, is represented by phagocytosis (nonspecific immunity *via* hemocytes) and respiratory burst (metabolic reactions following

TABLE III. Statistical comparisons (repeated-measure ANOVA with *F* and *P* values) of growth and fecundity between control *Lymnaea stagnalis* and snails exposed to 33 μg/L MC-LR

	Toxin Effect		Time Effect		Toxin × Time Effect	
	F	*P*	*F*	*P*	*F*	*P*
Shell size of juveniles	4.00	0.0668	1112	0.0001*	0.25	0.9607
Shell size of adults	0	>0.05	44.59	0.0001*	−2.511 E−14	1
Egg masses number	7.92	0.0078*	11.69	0.0001*	4.57	0.0002*
Egg number	3.74	0.0609	10.68	0.0001*	3.60	0.0002*

*Significant at $P < 0.05$, DDL = 38.

stimulation of phagocytic hemocytes) (Russo and Lagadic, 2004). They are probably also implicated to counteract the adverse effects of cyanotoxins and may be less efficient in immature snails. Numerous authors have demonstrated the biodegradation of microcystin by aquatic bacteria (Jones and Orr, 1994; Jones et al., 1994; Rapala et al., 1994), and also its biotransformation that reduces toxicity (Lam et al., 1995; Cousins et al., 1996). Up to now, no degradation of microcystin has been demonstrated in gastropods (*via* bacteria or not), even if some herbivorous species are known to harbor bacteria in their gut. The question is to know if some of these bacteria are able to degrade microcystin in the gut of lymneids. If possible, one can hypothesize that a partial degradation of microcystin may be realized in *L. stagnalis*, and with a higher rate in case of adults *versus* juveniles.

According to our results, the fecundity of polluted adults (eggs and egg masses number) is decreased to half compared with that of the controls, while survival (no mortality), growth, and locomotion of *L. stagnalis* are not significantly altered by the occurrence of MC-LR in water (Tables I–III). The mean shell growth rate of juveniles and adults was respectively 1.21 $\pm$ 0.04 mm and 0.15 $\pm$ 0.02 mm per week. Egg laying was not observed for snails exposed as juveniles, which were not sexually mature at the end of the experiment. Locomotory activity was significantly superior in case of juveniles *vs* adults (Table III). Resource allocation pattern is different according to the age (physiological status) of the snails. While energy is channeled toward somatic growth in juvenile snails, energy in adults is mainly channeled to egg production, even if they continue to grow but to a lesser extent. The decrease of fecundity in case of adults exposed to MC-LR may be interpreted as a consequence of a reallocation of a part of energy, normally channeled to reproduction, toward the immune system. In case of juveniles, long-term studies are necessary to verify the absence of pathology on reproduction (development of gonad and accessory sexual organs, age/size at sexual maturity if acquired. . .).

To conclude, we assessed the possible accumulation of MC-LR in juvenile and adult *L. stagnalis* following direct ingestion of aqueous MC-LR, which resulted in a strong decrease of egg production in adults. From our findings, we can hypothesize that the occurrence of dissolved microcystin in the field may have a delayed effect on the dynamics of natural snail populations *via* the reduction of their reproductive potential and brood size. In temperate regions, the increase of aqueous microcystin concentration following cyanobacterial lysis takes place in late summer and beginning of autumn, a period that coincides with the second breeding season for many species of freshwater pulmonates, including the genus *Lymnaea* (Russel-Hunter, 1961). In this context and because of the delayed effect, to show the toxic effect of dissolved microcystin on natural populations of gastropods will be difficult and needs long-term investigations that take into account the life-cycle patterns of snail species and the time variations of dissolved microcystin concentrations.

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