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Interactions between cyanobacteria and Gastropods I. Ingestion of toxic *Planktothrix agardhii* by *Lymnaea stagnalis* and the kinetics of microcystin bioaccumulation and detoxification

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Abstract

The last two decades have been marked by an increasing occurrence of toxic cyanobacterial blooms in aquatic ecosystems. These pose an expanding threat to the environment and to human health. Among the intracellular toxins produced by cyanobacteria, microcystins (hepatotoxins) are the most frequent and widely studied. As an ubiquitous herbivore living in eutrophic freshwaters, the freshwater snail *Lymnaea stagnalis* (Gastropoda: Pulmonata) is particularly exposed to cyanobacteria. The toxic filamentous *Planktothrix agardhii* is common in temperate lakes and is, therefore, a potential food resource for gastropods. We have studied the consumption of *P. agardhii* by *L. stagnalis* juveniles and adults in the presence or absence of non-toxic food (lettuce) over a 5-weeks period. Intoxication was followed by a 3-week detoxification period when snails were fed only on lettuce. The kinetics of microcystin accumulation and detoxification in the gastropods were established using the ELISA analytical method. The results showed an ingestion of toxic *P. agardhii* by *L. stagnalis*, even in the presence of lettuce, and the absence of food selection regardless of the age of the snails. Juveniles and adults consumed the same number of cells per millilitre and consumption was proportional to food availability. On average, 63% of cyanobacteria available were taken up during the first 24 h. After 5 weeks of intoxication, 61% of the toxins present in the ingested cyanobacterial cells had accumulated in snail tissues (95% in the digestive–genital gland complex) with a concentration up to 80.4 ± 4.9 μg g DW^{-1}. Toxin accumulation was greater in the gastropods fed on *P. agardhii* alone than those fed on the mixed diet, and was also greater in juveniles than in adults. After the removal of toxic cyanobacteria, detoxification was rapid: 64% of the toxins disappeared from snail tissues during the first week, but microcystins were still detected after 3 weeks (on average, 3.5 ± 0.9 μg g DW^{-1}). These results are discussed in terms of potential contamination to the food web.

Keywords: *Lymnaea stagnalis*; Cyanobacteria; Microcystins; Bioaccumulation; Detoxifcation

1. Introduction

Cyanobacteria can form massive blooms in freshwater bodies and produce a wide range of toxins such as hepatotoxins, neurotoxins and lipopolysaccharides. Hepatotoxins have a more widespread occurrence and are found in 40–75% of cyanobacterial blooms (Chorus and Bartram, 1999). The most studied hepatotoxins are the microcystins, cyclic heptapeptides of which 80 variants have been identified (Dietrich and Hoeger, 2005). Contamination of organisms can occur by exposure to soluble toxins, direct consumption of cyanobacterial cells and by consumption of contaminated prey. Microcystins have been recognized to accumulate and induce extensive damage in several organisms including zooplankton, bivalves and fish, after ingestion of cyanobacterial cells (for review: Zurawell et al., 2005).

Freshwater gastropods have rarely been considered in toxic cyanobacteria studies. However, these organisms represent an important part of freshwater macroinvertebrate biomass. They are important links between primary producers and higher consumers, and they often play key roles in structuring aquatic communities (Habdija et al., 1995). Recently, pathogenic effects of dissolved microcystin-LR on life-traits have been demonstrated in laboratory experiments on two gastropod species: the prosobranch *Potamopyrgus antipodarum* has shown a decrease in survival, growth and fecundity (Gérard

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and Poullain, 2005) while the pulmonate *Lymnaea stagnalis* has shown a decrease in fecundity (Gérard et al., 2005).

Freshwater pulmonates like lymnaeids inhabit shallow littoral zones and are predominantly herbivores. They are considered as indiscriminate grazers searching for food in the entire water column and on various substrates of the littoral and adapt their diet to the relative abundance of available resources (Bovbjerg, 1968; Reavell, 1980; Brendelberger, 1997). As cyanobacteria can dominate phytoplankton community and colonize littoral waters in bloom periods, it is therefore relevant to ask whether these grazers would consume large quantities of toxic cyanobacteria and whether they would be affected by this consumption. The few field studies which have included gastropods (Kotak et al., 1996; Zurawell et al., 1999; Ozawa et al., 2003; Chen et al., 2005) have hypothesized a consumption of toxic cyanobacteria following a positive relationship found between toxin concentration in phytoplankton and microcystin accumulation in gastropod tissues.

L. stagnalis is characteristic of eutrophic aquatic systems (Clarke, 1979) which are more prone to cyanobacterial blooms. *Planktothrix agardhii*, a filamentous planktonic species is the most common cyanobacterium in eutrophic lakes of temperate areas (Scheffer et al., 1997; Brient et al., 2004) and has a greater cellular toxin production than other colonial or unicellular species (Christiansen et al., 2003). Although this species is planktonic, wind events have been shown to concentrate filaments in littoral zones. Indeed, Webster and Hutchinson (1994) predict that a blue-green population should be more strongly concentrated towards the downwind end of a lake. This prediction is in accord with measured distributions in lakes. Densities of toxic cyanobacteria can thus become very high in shallow waters and with the filaments trapped and accumulating in dense macrophytes, on rocks and littoral sediments, increasing the probability of grazing by snails.

This study is the first part of a research program on freshwater cyanobacteria–gastropod interactions and examines the potential of *L. stagnalis* to ingest *P. agardhii* and to accumulate microcystins. The second part of our work focuses on the negative impact of toxic cyanobacteria ingestion on the life traits of gastropods (survival, growth, fecundity), and the plasticity of the response according to their development stage (juveniles or adults). Results will be presented in a separate publication.

Consumption of cyanobacterial cells by gastropods in the presence or not of non-toxic food was monitored during 5 weeks to study the feeding behaviour of *L. stagnalis*. Toxin production by *P. agardhii* was evaluated to assess the quantity of microcystins ingested by snails. Additionally, we investigated the potential of *L. stagnalis* to accumulate microcystins by establishing the kinetics of accumulation and detoxification of microcystins in gastropod tissues during a 5-weeks intoxification period followed by a 3-weeks detoxification period, and by calculating the ratio of accumulated/ingested toxins.

We focused the discussion on the potential accumulation of cyanotoxins by lymnaeid snails in the field and contamination risk for the food web, since these gastropods are consumed daily by numerous invertebrates (crayfish, leeches, aquatic insects as adult coleopterans or larval tabanids) and vertebrates (fish, waterfowl) (for review: Michelson, 1957), which in turn are consumed by aquatic or terrestrial predators like fish, amphibians, musk rats and birds.

2. Material and methods

2.1. Biological material

Prior to the experiments, juvenile and adult *L. stagnalis* (14 ± 1 and 25 ± 1 mm shell length, respectively) were acclimatized at constant temperature ($20 \pm 1\,^{\circ}C$) and photoperiod (12-h light:12-h dark cycle) and fed on dried lettuce ad libitum during 7 days. The filamentous cyanobacterium *P. agardhii*, originating from the recreational watersport site at Viry (Essone, France), was maintained in a modified medium (20 mL of liquid BG11 per litre of dechlorinated water). Cyanobacteria were placed in an incubation room at constant temperature ($25 \pm 2\,^{\circ}C$) and photoperiod (12-h light:12-h dark cycle) at an irradiance of $40\,\mu E\,m^{-2}\,s^{-1}$. The algal concentrations of 200,000 cells mL^{-1} were provided twice a week to the gastropods. These algal suspensions produced dmMC-LR and MC-RR (detected by HPLC–MS), with a total concentration of $5\,\mu g\,L^{-1}$ expressed as microcystins-LR equivalents (MC-LReq) and measured by high-performance liquid chromatography (HPLC, Section 2.3.2). The density of cyanobacteria used in this study is similar to that commonly found, and often exceeded in natural systems worldwide (Chorus and Bartram, 1999).

2.2. Experimental setup

Following the period of acclimatization, snails were divided into four groups according to age and diet for a 5-weeks intoxication experiment: (1) cyanobacterial suspension as the only food source for juveniles (juv cyano) and (2) for adults (ad cyano), (3) a mixture of cyanobacterial suspension and lettuce ad libitum for juveniles (juv cyano + let) and (4) for adults (ad cyano + let). Each group consisted of 35 replicates, i.e. 35 isolated individuals, in glass containers of 15 mL for each juvenile and 40 mL for each adult. Preliminary observations showed that gastropods consumed the *P. agardhii* suspension of 200,000 cell mL^{-1} in 3 days, hence cyanobacteria suspension was renewed twice a week. Control glass containers of 15 and 40 mL were filled in the absence of gastropods, with cyanobacteria suspension with and without lettuce during 5-weeks. Preliminary experiments performed over 20 days on 30 replicates with and without lettuce showed that the *P. agardhii* growth was similar and constant. It was assumed that two replicates per treatment were sufficient to measure cyanobacterial growth, and the influence of the presence of lettuce on the development of cyanobacteria, as the conditions for growth (light, temperature, nutrient) were identical. After the intoxication period, all gastropods were fed solely on lettuce ad libitum and maintained in dechlorinated non-toxic water during a 3-weeks detoxification period.

2.3. Cyanobacterial cells and microcystin ingestion estimates

2.3.1. Growth and ingestion model

To estimate the number of cells ingested by *L. stagnalis*, cyanobacterial densities were determined daily on microscope with a Nageotte chamber. The total length of all *P. agardhii* filaments in a 50 μL volume was measured and expressed in cells mL^{-1} assuming an average length of one cell of *P. agardhii* was 3.12 μm, based on 50 measurements. These cell counts were made in all groups and controls for the 5-weeks intoxication period (10 renewals of suspension). Cyanobacterial growth rate (μ) was determined from the rate of change in the cyanobacterial biomass (B) over time in the controls, as follows:

$$\frac{dB}{dt} = \mu B \quad (1)$$

The time interval used in this study was one day and we used the discrete time form of its precedent transition equation to estimate the daily growth rate (μ) as follows:

$$B_{t+1} = B_t(1 + \mu) \quad (2)$$

where B_t and B_{t+1} are, respectively, the biomass of cyanobacteria at time t and $t+1$ (in cells mL^{-1}). An assumption was made that μ was not influenced by the presence or absence of a gastropod. Hence, the daily gastropod ingestion rate of cyanobacteria in the treatments without lettuce was obtained by including the ingestion as a loss term (S) in Eq. (2) as follows:

$$B_{(S)t+1} = B_{(S)t} \times (1 + \mu - S) \quad (3)$$

where $B_{(S)t}$ and $B_{(S)t+1}$ are, respectively, the biomass of cyanobacteria at time t and $t+1$ (in cells mL^{-1}) in the treatments with snails and without lettuce. The total number of cells per millilitre ingested by snail per day was obtained by multiplying the cyanobacterial biomass $B_{(S)t}$ by the ingestion rate (S).

As preliminary tests revealed that the addition of lettuce introduced and enhanced the development of microorganisms that led to a cyanobacterial loss, we also estimated the quantity of cells ingested by each snail in the presence of lettuce in their diet. Several assumptions were required to quantify this: (1) that cyanobacteria have the same growth rate (μ) in the presence of lettuce or not, (2) the snails do not consume microorganisms and (3) the daily loss rate of cyanobacteria due to the presence of lettuce (L) is independent of the presence of a snail. We first estimated L as follows:

$$B_{(L)t+1} = B_{(L)t} \times (1 + \mu - L) \quad (4)$$

where $B_{(L)t+1}$ and $B_{(L)t}$ are, respectively, the biomass of cyanobacteria at time t and $t+1$ (in cell mL^{-1}) in the controls with lettuce and no snails. The daily snail ingestion rate of cyanobacteria in the presence of lettuce (SL) was obtained by considering the presence of microorganisms, as follows:

$$B_{(SL)t+1} = B_{(SL)t} \times (1 + \mu - L - SL) \quad (5)$$

where $B_{(SL)t+1}$ and $B_{(SL)t}$ are, respectively, the density of cyanobacteria at time t and $t+1$ (in cell mL^{-1}) in the treatments with lettuce and snails. The total number of cells per millilitre ingested daily by a snail in presence of lettuce was estimated by multiplying $B_{(SL)t}$ by SL.

The percentage of cells ingested by snails compared to those available (%conso) in the absence of lettuce was calculated as follows:

$$\%\text{conso} = 100 \times \left[\frac{S}{1 + \mu}\right] \quad (6)$$

The %conso in the presence of lettuce was calculated with SL instead of S in Eq. (6). Ingestion of cyanobacteria by snails was estimated for each group and expressed in several ways: as the average total number of cells ingested per snail and per renewal of suspension (twice a week), as the average number of cells ingested per snail and per millilitre of available suspension twice a week, and as the average total number of cells ingested per dry weight of snail and per renewal of suspension, in %conso per day or in average %conso per 3 days.

2.3.2. Ingestion of microcystins: HPLC analysis

Total microcystin content ingested per snail per week was estimated from the cyanobacterial cell toxin concentration and the average number of cyanobacterial cells ingested. Toxin concentration in cyanobacterial cells was determined each week with a HPLC with diode array detection (HPLC-DAD) and a variable-wavelength UV detector operating at 238 nm. Prior to HPLC analysis, cells harvested by filtration (nylon cloth, 2 μm pore size) were suspended in 0.5 mL of 85% methanol in water and centrifuged at 7000 × g for 7 min. The volume injected was 20 μL with a flow rate of 1 mL min^{-1}. The separation was performed on a microspher C18 reverse-phase column (3 μm) under isocratic conditions with a mobile phase of 10 mm ammonium acetate and acetonitrile (7.4:2.6) for 10 min. As microcystin-LR was the standard used, concentration was expressed as microgram cellular MC-LReq per litre of suspension. Microcystin contents estimated were combined with cell counts to derive a relation between algal density and microcystin production to obtain the amount of toxins produced by one cell of *P. agardhii*.

2.4. Quantitative analysis of MC-LReq in exposed snail tissues and quality control of microcystin measurement

Every week, two snails were randomly chosen from each group and were starved for ca. 24 h to empty their gut contents (Carriker, 1946) to ensure that the microcystin measurement reflected only assimilated toxins and did not include microcystins in the undigested filaments of *P. agardhii* or microcystins in the gut. Snails were removed from their shells, freeze-dried and weighed prior to microcystin analysis. This analysis was performed by immuno-assay with an ELISA Microcystin Plate Kit (Envirologix Inc.), which detects all of the 6 purified hepatotoxins of common bloom-forming cyanobacteria, especially MC-LR and MC-RR, from 0.05 μg L^{-1} threshold and to the nearest 0.01 μg L^{-1} (Codd et al., 1997; Gilroy et al., 2000). All microcystins of the *P. agardhii* strain used were thus detected

and expressed in MC-LReq using MC-LR, given by the supplier as standard. Microcystins were extracted with 2 mL of 100% methanol (Codd et al., 1997). Each snail was crushed in 1 mL of 100% MeOH and then crushed again after 12 h at 4 °C with 1 mL MeOH added. For the immuno-assay analysis the extract was diluted with water to less than 5% MeOH (Beattie et al., 1998). At the end of both the intoxication and detoxification periods, microcystin analysis was performed separately on the cephalopedial zone and the digestive–genital gland complex. The aim was to determine the relative importance of visceral mass and foot muscle tissue in the accumulation. Microcystin contents in snail tissues are expressed in $\mu g\,g\,FW^{-1}$ (fresh weight) and in $\mu g\,g\,DW^{-1}$ (dry weight) according to the relation previously established: FW = 7.47 × DW ($n = 30$, R Pearson = 0.94, $P < 0.05$). The values were calculated by taking into account extraction recovery and possible matrix-induced signal enhancement or suppression with the ELISA test because of unspecific binding to and/or denaturing of the antibodies. Control snails, free of microcystins, were freeze-dried and homogenized in a mortar, spiked with MC-LR standard ($5\,\mu g\,g^{-1}$) (Dionisio Pires et al., 2004). The extraction was performed as described previously and the recovery for the extraction was calculated. The matrix effect (i.e. effect of snail tissue) was checked by spiking control snails with MC-LR standard ($5\,\mu g\,g^{-1}$) and the response was compared to 100% methanol spiked with the same amount (Dionisio Pires et al., 2004). The average recovery was 72 ± 5.3% and matrix effect was negligible (from 0.05 to 4.8% of differences between matrix and methanol results with an average of 1.7 ± 0.4%). Similar results about the matrix effect with the ELISA test were observed by Ernst et al. (2005).

The percentage bioaccumulation (%acc) was calculated each week based on the ratio between the average quantity of MC-LReq accumulated in snail tissues between weeks 1 and n (in μg) and the estimated average quantity of MC-LReq ingested between weeks 1 and n (in μg). During the detoxification period, the percentage toxin elimination from snail tissues (%detox) was calculated between weeks by the expression:

$$\%\,\text{detox} = \frac{100 \times [\text{MC-LReq}_n - \text{MC-LReq}_{n+1}]}{\text{MC-LReq}_n} \quad (7)$$

2.5. Statistical analysis

Pearson's coefficient (R Pearson) was calculated to assess the existence of a correlation between: (1) the quantity of microcystins produced and the density of cyanobacterial suspensions ($n = 90$), (2) the fresh and dry weights of snails ($n = 30$). One analysis of covariance (ANCOVA) was performed on the cyanobacteria counts in the controls to compare their growth rates (μ) between weeks and volumes of culture medium ($n = 120$). Two-way analyses of variance (ANOVA) with repeated measures and the Tukey HSD multiple comparison test were performed to compare: (1) the total number of cyanobacterial cells and the number of cyanobacterial cells per millilitre ingested per snail, (2) the average %conso of snails over a 3 days period, (3) the total number of cyanobacterial cells ingested per dry weight of snails, (4) the quantity of microcystins bioaccumulated in $\mu g\,g\,DW^{-1}$, (5) the %acc, (6) the %detox. Differences were considered to be statistically significant at $P < 0.05$. Data are reported as means ± standard errors (±S.E.).

3. Results

3.1. Ingestion of P. agardhii cells and microcystins

The daily growth rate μ of *P. agardhii* was similar in the 15 or 40 mL containers and between weeks in the controls (cyanobacteria alone) (ANCOVA, $n = 120$, $P = 0.53$), and was $0.19 \pm 0.02\,day^{-1}$ (Fig. 1). When lettuce was present, cell density of *P. agardhii* was lower (Fig. 1), showing a loss of cyanobacteria to the introduced microorganisms which almost compensated for the growth rate. When snails were present, with or without lettuce, cyanobacteria density was lower than in their absence (Fig. 1), demonstrating the consumption of *P. agardhii* by *L. stagnalis*. In the 40 mL algal suspension, adults consumed 2.5 times more cells than juveniles in a 15 mL algal suspension over the 3 days, with and without lettuce (ANOVA $F_{3,20} = 434.3$, $P < 0.05$ and Tukey HSD, $P < 0.05$) (Table 1). When expressed in cell number per millilitre, consumption was similar in adults

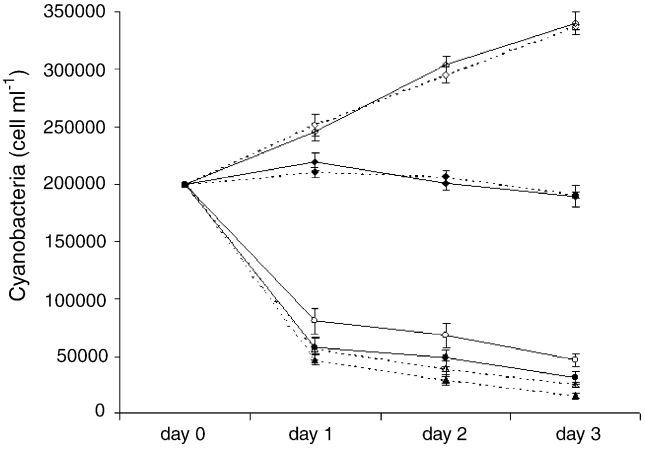

Fig. 1. Dynamics of *P. agardhii* populations during 3 days: alone (open diamond) and with lettuce (closed diamond), in the presence of snails as juveniles (triangle) and adults (circle) without (open) and with lettuce (closed) (dotted line for 15 mL and continuous line for 40 mL glass containers).

Table 1
Mean (±S.E.) ingestion of *P. agardhii* cells by *L. stagnalis* juveniles and adults over 3 days during the 5-weeks intoxication period in the presence or absence of lettuce (cells per snail, cells per snail per mL)

	Cells per snail	Cells per snail per mL
Juveniles fed on cyanobacteria (juv cyano)	3376000 ± 18000	225000 ± 1200
Juveniles fed on cyanobacteria and lettuce (juv cyano + let)	2882000 ± 124000	192000 ± 8000
Adults fed on cyanobacteria (ad cyano)	8428000 ± 160000	211000 ± 4000
Adults fed on cyanobacteria and lettuce (ad cyano + let)	7214000 ± 358000	180000 ± 9000

and juveniles (Tukey HSD, $P > 0.05$) but different according to the diet (ANOVA $F_{3,20} = 23.5$, $P < 0.05$). Snails with lettuce consumed less cells per millilitre than snails without lettuce, regardless of age (Tukey HSD, $P < 0.05$) (Table 1). This difference reflects the diminution of the availability of cyanobacteria due to the presence of lettuce. The mean %conso over 3 days, which takes account of the decline in available cyanobacterial cells for snails when microorganisms were present, was not significantly different between groups and weeks (ANOVA, respectively, $F_{3,20} = 2.3$ and $F_{4,20} = 1.3$, all $P > 0.05$). In each age class, it was similar in the presence or absence of lettuce (Tukey HSD, all $P > 0.05$), suggesting an absence of non-toxic food selection in juveniles and adults. In addition, the daily %conso changed over the 3 days during the intoxication period (ANOVA, $F_{2,108} = 91.4$, $P < 0.05$). It was significantly higher the first day (Tukey HSD, $P < 0.05$), $62.8 \pm 1.7\%$ for all groups, whereas it did not significantly differ during the remaining 2 days (Tukey HSD, $P > 0.05$), mean of $33.1 \pm 1.8\%$.

When normalised against dry weight of snails, total number of cells ingested differed according to age (ANOVA, $F_{3,20} = 97.6$, $P < 0.05$). Adults ingested in total 1.4 times more cells per gram of dry weight than juveniles (Tukey HSD, $P < 0.05$). Such differences are relevant for comparisons of the differences in bioaccumulation of toxins per gram of weight.

The total quantity of microcystins ingested was estimated from the production of MC-LReq by one *P. agardhii* cell (3×10^{-8} μg, $n = 45$, R Pearson = 0.9, $P < 0.05$). It was about 2.5 times higher for adults than for juveniles, in relation with the total number of cells ingested. At the end of the 5 weeks intoxication period, the total content of microcystins consumed was estimated for adults at 2.2 ± 0.0 μg ind^{-1} without lettuce and 1.9 ± 0.1 μg ind^{-1} with lettuce, and for juveniles at 0.9 ± 0.0 μg ind^{-1} without lettuce and 0.7 ± 0.0 μg ind^{-1} with lettuce.

3.2. Bioaccumulation and detoxification

Microcystin contents in snails increased steadily during the intoxication and reached a maximum of 80.4 ± 4.9 μg g DW^{-1} (10.8 ± 0.7 μg g FW^{-1}) after 5 weeks in the case of juveniles without lettuce (Fig. 2). Differences in MC-LReq contents per gram were significant between groups (ANOVA, $F_{3,20} = 24.8$, $P < 0.05$). Juveniles accumulated 2 times more toxins per gram than adults (Tukey HSD, $P < 0.05$), in spite of having consumed 1.4 times less cells per gram of dry weight (and 2.5 times less cells in total). Moreover, individuals fed on cyanobacteria alone accumulated more toxins per weight than those fed on the mixed diet (Tukey HSD, $P < 0.05$). The %acc, which takes into account differences in cell consumptions, varied between groups

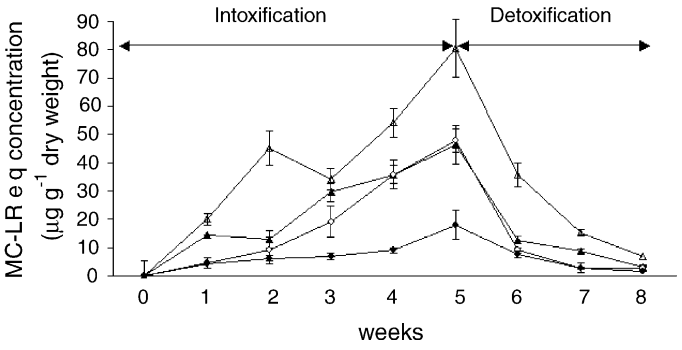

Fig. 2. Kinetics of MC-LReq in *L. stagnalis* tissues (μg g DW^{-1}) of juveniles (triangle) and adults (circle) fed on cyanobacteria without (open) and with lettuce (closed) during 5 weeks of intoxication, then fed on lettuce during 3 weeks of detoxification.

(ANOVA, $F_{3,20} = 7.1$, $P < 0.05$) and showed similar trends. In general, juveniles accumulated greater proportions of ingested microcystins than adults, and for both ages, snails fed on *P. agardhii* alone had a greater %accu than snails fed on mixed diet (Fig. 3). However, only the differences between adults fed on *P. agardhii* alone and the other groups were significant (Tukey HSD, $P < 0.05$). An average of 61% of total ingested toxins was accumulated by all gastropods after the intoxication.

In addition, more than 95% of accumulated toxins were located in digestive–genital gland complex (less than 5% in cephalopedial zone) (Table 2).

During the 3 weeks-detoxification period, mean microcystin contents declined in all groups from 48.0 ± 7.9 to 3.5 ± 0.9 μg g DW^{-1} (or from 6.4 ± 0.9 to 0.4 ± 0.1 μg g FW^{-1}). Adult snails fed on *P. agardhii* alone, which had accumulated the highest total quantity of toxins at week 5 (in the whole body and not per gram of weight), eliminated every week a greater proportion of the toxin contents

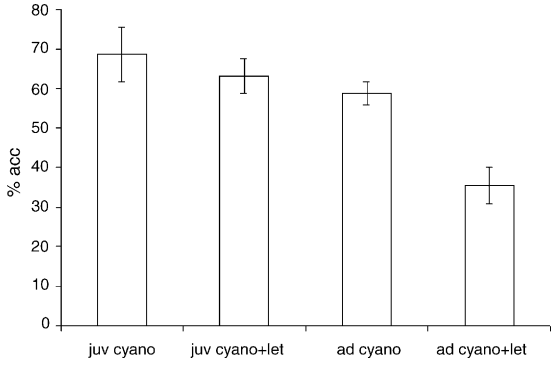

Fig. 3. Mean (±S.E.) percentage of ingested MC-LReq accumulated in *L. stagnalis* tissues (%acc) of juveniles (juv) and adults (ad) fed on cyanobacteria without (cyano) and with lettuce (cyano + let) during 5 weeks of intoxication.

Table 2
Mean (±S.E.) concentration of MC-LReq (μg g DW^{-1}) in *L. stagnalis* digestive–genital gland complex at the end of 5-weeks intoxication and 3-weeks detoxification periods according to the age and the presence of lettuce (see abbreviations in Table 1)

	juv cyano	juv cyano + let	ad cyano	ad cyano + let
End of intoxication	194.8 ± 13.5	170.5 ± 12.9	135.8 ± 1.4	35.8 ± 4.0
End of detoxification	22.1 ± 2.1	20.4 ± 4.9	9.4 ± 0.9	4.6 ± 0.5

Table 3
Mean (±S.E.) percentage of MC-LReq eliminated every week in *L. stagnalis* tissues (%detox) during the 3-weeks detoxification period, according to the age and the presence of lettuce (see abbreviations in Table 1)

Weeks	juv cyano	juv cyano + let	ad cyano	ad cyano + let
6	42.1 ± 2.4	76.9 ± 1.1	81.0 ± 0.9	54.4 ± 3.9
7	55.9 ± 2.3	31.0 ± 2.4	67.8 ± 5.6	63.3 ± 1.9
8	55.2 ± 2.1	63.9 ± 0.9	43.7 ± 5.0	37.9 ± 3.6

in their tissues than other groups (ANOVA, $F_{3,156} = 11.5$, $P < 0.05$ and Tukey HSD, $P < 0.05$), which showed similar percentages of detoxification (Tukey HSD, $P > 0.05$) (Table 3). Moreover, the percentage detoxification was significantly higher the first week: 63.6 ± 2.1% for all groups (ANOVA, $F_{2,156} = 19.7$, $P < 0.05$ and Tukey HSD, $P < 0.05$), whereas elimination of toxins did not significantly differ during the last 2 weeks, 54.5 ± 3.0 and 50.2 ± 2.9%, respectively (Tukey HSD, $P > 0.05$). At the end of the experiments, snails had eliminated on average 91.9 ± 0.5% of the accumulated toxins.

4. Discussion

The results show that *L. stagnalis* ingested hepatotoxic strains of *P. agardhii*, even in the presence of another non-toxic food source (lettuce). Therefore, no food selection by *L. stagnalis* occurred in contrast to some copepod and fish species which have been shown to select and ingest only the non-toxic food in laboratory experiments (for review: Zurawell et al., 2005). On average 63% of cyanobacterial cells were consumed during the first 24 h and 33% during the remaining 2 days. We observed that cyanobacterial filaments sank quickly to the bottom of containers after the renewals of suspension, forming a dense layer of food for snails. Sedimentation was probably due to the absence a buoyancy control necessary to access light since the light climate was homogenous in glass containers. Search for food by *L. stagnalis* is done by random movements (Bovbjerg, 1968) and the decrease of cyanobacteria consumption after 24 h may be a consequence of the decrease in encounter rates between snail and cyanobacterial cells. The search for food is accomplished by waving the head from side to side, and the tentacles are sensitive to food contact. As cyanobacteria were consumed, their concentrations decreased and the probability of detection by the snail was reduced.

In the presence of lettuce, cyanobacteria were consumed by snails, but *P. agardhii* availability was also decreased due to microorganisms. Snails consumed the same proportion of available cells as when fed only on cyanobacteria, but ingested a smaller total number of cells. As found by Sheerboom and Geldof (1978), the amount of food ingested is always related to the amount available and *L. stagnalis* fed almost continuously day and night without reaching a satiety threshold. Comparison between adults and juveniles showed that they consumed the same number of cells per millilitre. The feeding behaviour of *L stagnalis* was similar irrespective of age and of alternative non-toxic food source, the determining factor was the availability of cyanobacteria in the medium.

Following consumption of toxic cyanobacteria, lymnaeid snails rapidly accumulated microcystins readily detectable at the end of the first week of intoxication period (a maximum of 619 ng of MC-LReq in total body) and increasing with time (a maximum of 3.2 μg in total body after 5 weeks). Juvenile snails fed on cyanobacteria had the greatest microcystin concentration per gram at week 5: on average 80.4 ± 4.9 $\mu g\,g\,DW^{-1}$ (Fig. 2). A similar bioaccumulation was observed in the gastropod *Sinotaia histrica* after consumption of a toxic *Microcystis* strain (Ozawa et al., 2003). In Canadian lakes, Zurawell et al. (1999) reported similar or higher values of microcystin accumulation in three gastropod species (up to 140 $\mu g\,g\,DW^{-1}$ for *L. stagnalis*, 130 $\mu g\,g\,DW^{-1}$ for *Physa gyrina* and 40 $\mu g\,g\,DW^{-1}$ for *Helisoma trivolis*). They considered that the concentration of MC-LReq in tissues was correlated with toxins found in phytoplankton (up to 1526 $\mu g\,g\,DW^{-1}$ in hypereutrophic lakes), and not with dissolved toxins (up to 1.2 $\mu g\,L^{-1}$). In laboratory experiments, *L. stagnalis* exposed for 6 weeks to 33 $\mu g\,L^{-1}$ dissolved MC-LR accumulated only a maximum of 0.06 $\mu g\,g\,DW^{-1}$ (Gérard et al., 2005). For comparison, *L. stagnalis* in this study, exposed to 5 $\mu g\,L^{-1}$ of intracellular MC-LReq, accumulated almost 1300 times more toxins after 5 weeks. It appears from these results that gastropods accumulated microcystins mainly by grazing toxic phytoplankton, and to a lesser extent, via uptake of dissolved toxins.

Based on the percentage of accumulation, an average of 61% of total ingested toxins was accumulated by all gastropods after the intoxication. However, microcystin concentrations found in *L. stagnalis* were probably underestimated in the present experiment, due to the limitation in microcystin extraction from snail tissues. Indeed, an undetermined part of microcystins, i.e. those covalently bound to protein phosphatase, are not extractable by methanol and not detectable by the ELISA test. Thus, the microcystin concentrations we reported in tissue samples correspond to free and metabolised microcystins (i.e. conjugated with glutathione and cysteine, with which microcystin antibodies of the ELISA test crossreact by immunoaffinity (Metcalf et al., 2000)). The existence of non-extractable microcystins has been demonstrated in some bivalves by several authors (Williams et al., 1997; Dionisio Pires et al., 2004; Dietrich and Hoeger, 2005). Less than 0.1% of the total microcystins was extractable with methanol in saltwater mussels (Williams et al., 1997). In zebra mussels, covalently bound MC-LR was generally lower than free unbound MC-LR but could reach 62% of free MC-LR (Dionisio Pires et al., 2004). Further investigations are required to estimate the percentage of covalently bound and free microcystins in snail tissues.

The 39% of total ingested microcystins that were not measured in *L. stagnalis* are thought to have been partly: (i) eliminated in the gizzard and cecal string fraction of the faeces (undigested cells) during the first hours post ingestion. According to Zurawell et al. (2006), 57% of the initial microcystin concentration are found in this faeces fraction of *L. stagnalis* within 8 h after removal from microcystin-containing cyanobacteria exposure. Other possible mechanisms by which ingested

microcystins escaped measurement include: (ii) entering the digestive gland, where intracellular digestion of cyanobacteria occurred, followed by excretion in the digestive gland fraction of the faeces (Carriker, 1946; Zurawell et al., 2006); (iii) entering in the digestive gland and accumulating through binding covalently to protein phosphatase enzymes and thus being undetectable (Dietrich and Hoeger, 2005).

The major accumulation site of microcystins in both experimentally exposed and wild invertebrates and vertebrates is the digestive gland (or liver) (Vasconcelos, 1995; Cazenave et al., 2005). According to Chen et al. (2005), the mean distribution of microcystins in the gastropod *Bellamya aeruginosa* is as follows: 64.5% in the digestive gland (4.4 μg g DW^{-1} MC-LReq), 24.8% in the digestive tractus (1.7 μg g DW^{-1} MC-LReq), 10.6% in the genital gland (0.7 μg g DW^{-1} MC-LReq) and 0.2% in the foot (0.05 μg g DW^{-1}). In this study, more than 95% of accumulated MC-LReq is detected in the digestive–genital gland complex of *L. stagnalis*, with an average concentration of 135 μg g DW^{-1} at the end of intoxication period.

Bioaccumulation capacity showed two trends according to age and diet: (1) juveniles accumulated a greater proportion of ingested toxins than adults (66% versus 47%), (2) lymnaeids which received only cyanobacteria had a greater accumulation than those fed on cyanobacteria with lettuce (64% versus 50%). Moreover, despite a larger intake of toxic food per body weight, adult snails had less amounts of toxins per gram, indicating they were more efficient in detoxifying and/or excreting the toxins than juveniles. Detoxification processes have been shown to occur in various organisms (e.g., plants, invertebrates and vertebrates) and allow organisms to survive under cyanobacterial stress (for review, see Cazenave et al., 2006). Accumulated microcystins can be metabolized into less harmful compounds after conjugation with glutathione via glutathione-*S*-transferase or glutathione peroxidase, resulting in microcystin excretion or physiological degradation. Previous studies, reported in Cazenave et al. (2006), have demonstrated that toxic cyanobacteria induce the production of reactive oxygen species (ROS) in relation with the immunological system. This oxidative stress is known to be reduced by the activity of antioxidant enzymes, such as glutathione peroxidase. The higher accumulation of cyanotoxins in juvenile (versus adult) *L. stagnalis* may be due to the less well developed, and therefore less competent, immune system (Dikkeboom et al., 1985), with a consequently less efficient detoxification system. Moreover, the resource allocated to detoxification processes, which has a high energy cost, should be derived from a common pool of limited resources used by all fitness-associated traits (Rigby and Jokela, 2000). Thus, snails which had received lettuce with cyanobacteria had a lower accumulation probably due to the enhanced energy uptake allowing a more efficient detoxification. The consequences of toxic cyanobacteria consumption in terms of impact on life traits and energy allocation according to the age and the food diet in *L. stagnalis* will be reported in a separate publication (in preparation).

As a consequence of detoxification processes in organisms exposed to toxic cyanobacteria, the potential contamination of the food web may be thought to be limited. At present, few toxicological studies (Laurén-Määttä et al., 1995; Engström-Öst et al., 2002; Ibelings et al., 2005) have investigated the transfer of microcystins in aquatic food web, and zooplankton and bivalves have mainly been considered as toxin vectors, not freshwater gastropods. Ibelings et al. (2005) showed a transfer of microcystins without biomagnification from zooplankton and zebra mussel to fish in a lake in the Netherlands. In the laboratory, accumulation of hepatotoxic nodularin was demonstrated in shrimps and three-spined sticklebacks fed on cyanobacteria-fed copepods (Engström-Öst et al., 2002). In this study, due to the elimination of 64% of the MC-LReq content in *L. stagnalis* tissues during the first week free of cyanobacteria, and of 92% after 3 weeks, the risk of toxin transfer to gastropod predators in the field is probably small outside bloom events. However, this risk remains as microcystins were still detectable in snail tissues after 3 weeks of detoxification (on average, 3.5 ± 0.9 μg g DW^{-1}). A similar detoxification efficiency is also demonstrated by Zurawell et al. (2006): the cumulative microcystin loss from *L. stagnalis* was 95% at 22 °C (80% at 10 °C) after 6 days following the removal from toxic cyanobacteria exposure, and 99.5% at 22 °C (97.5% at 10 °C) after 30 days.

However, as gastropods are generally common and abundant in fresh waters (Habdija et al., 1995) and are consumed by various invertebrate and vertebrate predators (for review, see Michelson, 1957), the accumulation of microcystins in their body could lead to a significant contamination of aquatic and terrestrial food webs. Moreover, the risk of toxin transfer to higher trophic levels is probably much higher in natural conditions for two reasons. Firstly, the accumulation reported in this experiment is underestimated due to the absence of detection of covalently bound microcystins in snails. Secondly, the concentration of cyanobacterial suspensions in this study was 200,000 cell mL^{-1}, which is similar if not less than those regularly observed in lakes, particularly in eutrophic waters (Chorus and Bartram, 1999). According to Brient et al. (2004), 70% of monitored lakes in Brittany (France) reach a cyanobacterial density between 100,000 and 5 millions cell mL^{-1} during the summer period. *P. agardhii* is a common microcystin producer in the Northern hemisphere (Scheffer et al., 1997) and may have an extended proliferation period, from April to October (Chorus and Bartram, 1999), or even persist perennially for many years by maintaining minimal density during winter (Briant et al., 2002). Hence, when cyanobacteria dominate the phytoplankton community for an extended period in eutrophic lakes, the quantity of toxic cells ingested by gastropods is probably far higher than in this experiment, resulting in a more important toxin accumulation in the field as suggested by Zurawell et al. (1999), especially in bloom periods. Consequently, the increased accumulation may delay the detoxification period and lengthen its duration.

To evaluate the risk of cyanotoxin transfer in the field, based on the age-dependant differences in microcystin accumulation by snails demonstrated here, we need to consider the life cycle of freshwater gastropods and the structure of their populations and communities during the blooms. Despite the high intraspecific interpopulation plasticity of freshwater gastropods and the

numerous life cycle patterns described in temperate regions (Calow, 1978), pulmonates are generally annual, univoltine and semelpare (even if *L. stagnalis* is among the few pulmonate species able to live for 2 years), whereas prosobranches tend to be perennial and iteroparous. The main breeding season takes place in late spring or early summer, and coincides with the beginning of the cyanobacterial population maxima. Most gastropods are consequently exposed to cyanobacteria from birth, when they are the most vulnerable to the predation pressure which focuses on neonates and juveniles, whereas the mortality of adults is mostly related to the reproductive effort (Calow, 1978). Moreover, juveniles, that accumulate more toxins per weight than adults, are probably more susceptible to the toxicity of cyanobacteria, with consequences for their life traits. Results on the impact of toxic cyanobacteria ingestion by *L. stagnalis* on survival, growth, fecundity and locomotion will soon be published in a separate publication. To complete this experimental research program, long term investigations are required in the field to demonstrate the cyanotoxin vector role of freshwater gastropods and the transfer patterns through the food web.

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