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Uptake and depuration of the heptapeptide toxin microcystin-LR in *Mytilus galloprovincialis*

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Abstract

Mussels (*Mytilus galloprovincialis* Lamarck) were exposed to the toxic cyanobacterium *Microcystis aeruginosa* Kutz. emend Elkin for 16 days in order to study bioaccumulation of the cyclic heptapeptide toxin microcystin-LR. After this time period the animals were fed marine phytoplankton for 2 weeks and the amount of microcystin in the mussels was monitored again. During the exposure phase mussels attained a maximum of 10.5 μg of toxin per g dry mussel weight at day 10. Microcystin was detectable after the second day of exposure. The percentage of toxin that was taken up relative to the total amount administered varied from 24.1% to 54.8%. When the mussels were fed marine phytoplankton there was a 50% decrease in the amount of detectable toxin in the mussels within 2 days. This was followed by a slight increase in toxin levels over 5 days with no microcystin-LR being detected at day 13. The major part of the toxin (96%) was found in the digestive gland + stomach while the gills, muscle, foot, and other organs combined had less than 4% of the total toxin. The results presented here show that cyanobacteria blooms present in estuaries where bivalves are growing naturally constitute a health hazard in that these organisms can retain microcystins and transfer them through the food chain.

Keywords: Microcystin-LR; Cyanobacteria; *Microcystis*; *Mytilus galloprovincialis*; Depuration

1. Introduction

Toxic cyanobacteria blooms are a worldwide problem (Watanabe and Oishi, 1982; Skulberg et al., 1984; Hawkins et al., 1985; Repavich et al., 1990; Carmichael and Falconer, 1993) that occur more frequently in freshwater ecosystems. Nevertheless, cyanobacteria can resist higher salinities ranging from brackish to marine to hyper-

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saline i.e., in the Baltic Sea (Sivonen et al., 1989) or in estuaries of some rivers (Falconer et al., 1992; Vasconcelos, 1994). The production of toxins by these organisms, especially the hepatotoxic peptide nodularins from *Nodularia spumigena* (Sivonen et al., 1989) and microcystins from genera such as *Microcystis*, *Anabaena* and *Oscillatoria* (Krishnamurthy et al., 1986; Sivonen, 1990; Luukkainen et al., 1993) poses a serious problem to human health if these toxins are ingested in the drinking water or accumulated by organisms that form part of the human food chain.

Toxic marine phytoplankton, especially dinoflagellates and diatoms, produce neurotoxins that may be accumulated by several organisms, and the transfer of the toxins through the food chain may reach humans. Human intoxications may be lethal depending on the dose of the toxin ingested. Molluscs are the most frequent organisms that accumulate these toxins, causing severe human intoxications such as Paralytic Shellfish Poisoning — PSP — (Prakash et al., 1971), Diarrhetic Shellfish Poisoning — DSP — (Kat, 1979), Amnesic Shellfish Poisoning — ASP — (Perl et al., 1990) and Neurotoxic Shellfish Poisoning — NSP — (Morris et al., 1991).

Not many studies have involved cyanobacteria toxin accumulation by molluscs perhaps for the main reason that freshwater molluscs are not usually consumed by humans. Nevertheless, Eriksson et al. (1989) found that the freshwater mollusc *Anodonta cygnea* can accumulate high levels of microcystins produced by *Oscillatoria agardhii* and not suffer any visible damage. Lindholm et al. (1989) also reported accumulation of hepatotoxic peptides in swan mussels (*A. cygnea*) from a natural water sample containing *O. agardhii*. More recently Falconer et al. (1992), using an edible mussel, *Mytilus edulis*, showed that they accumulate nodularin during high densities of *Nodularia spumigena* in the Peel Inlet estuary.

Most of the large Portuguese rivers (Minho, Douro, Tejo, Guadiana) develop toxic cyanobacteria blooms and eventually these blooms reach an estuary. This is especially true in the North-Iberian Minho river. Blooms in this river develop in some Spanish reservoirs and then float downstream till they reach the estuary where they can remain intact for several days (Vasconcelos, 1994). Mussels, cockles and other bivalves are exposed to these toxic organisms and can possibly accumulate cyanotoxins, especially microcystins.

This paper studies the uptake and depuration of the hepatotoxin microcystin-LR (MCYST-LR), produced by a toxic strain of *M. aeruginosa* — IZANCYA2 — in the mussel *M. galloprovincialis*.

2. Material and methods

Mussels were collected from an intertidal population of *Mytilus galloprovincialis* Lamarck on the shore of Foz do Douro, Porto, Portugal in an area not exposed to cyanobacteria toxins.

They were acclimated to laboratory conditions (16°C, 35‰) over a 4-week period. Natural sea water was filtered and sterilized using UV light and was changed every second day. They were fed daily on a mixture of *Tetraselmis suecica* and *Nitzschia* sp. (10^5 cells/ml). These two marine algae were cultured in nonaxenic batch cultures using

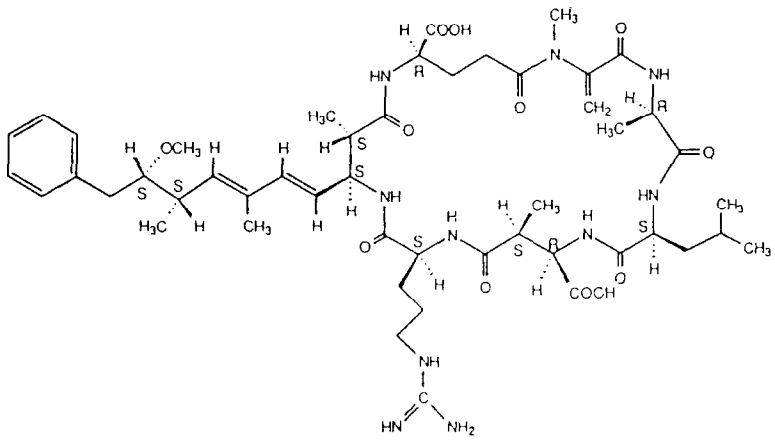

Fig. 1. Structure of microcystin-LR isolated from the toxic *Microcystis* strain IZANCYA2.

a medium described by Fabregas et al. (1984) that was prepared in filtered, sterilised natural seawater. They were grown under continuous white fluorescent light (Philips, 50 W) at 16°C with continuous aeration. A toxic strain of *Microcystis aeruginosa* — IZANCYA2 — was cultured in Z8 medium (Kotai, 1972) using the same conditions as those described for the marine algae. This strain was isolated from Lake Mira (Portugal) on 13 April 1991. It has an intraperitoneal mouse LD_{50} of about 7.5 mg/kg. This *Microcystis* strain produces MCYST-LR (Fig. 1) as its major toxin (95%), and low amounts of MCYST-LA and [D-Asp^3]-MCYST-LR. The isolation and assignment of the structures of these toxins was done according to the methods described in Vasconcelos et al. (1993). These results will be published elsewhere.

2.1. Uptake experiment

One-hundred-and-twenty mussels (length, 3.461 ± 0.124 cm; total body weight, 5.575 ± 0.598 g; dry weight, 0.152 ± 0.035 g) were held in each of three tanks with 20 l of natural seawater and a 2-cm-thick gravel bed. Water was continuously aerated and changed completely every second day. These mussels were fed daily the toxic *Microcystis* strain (1×10^5 cells/ml) for 16 days. The cell density is in the range found for blooms of *M. aeruginosa* in Portuguese freshwaters. During this exposure period dead animals were removed. A sample of 20 mussels was taken for toxin analysis on the first and second day and then every second day until day 16. The soft tissues were removed, immediately frozen and then freeze-dried. Daily samples of the toxic cyanobacteria culture used as food were also taken, frozen and then freeze-dried for toxin quantification. On day 16 of this experiment a final sample of 30 mussels was taken, dissected and the organs pooled (digestive glands + stomach, foot, muscle, gills, rest).

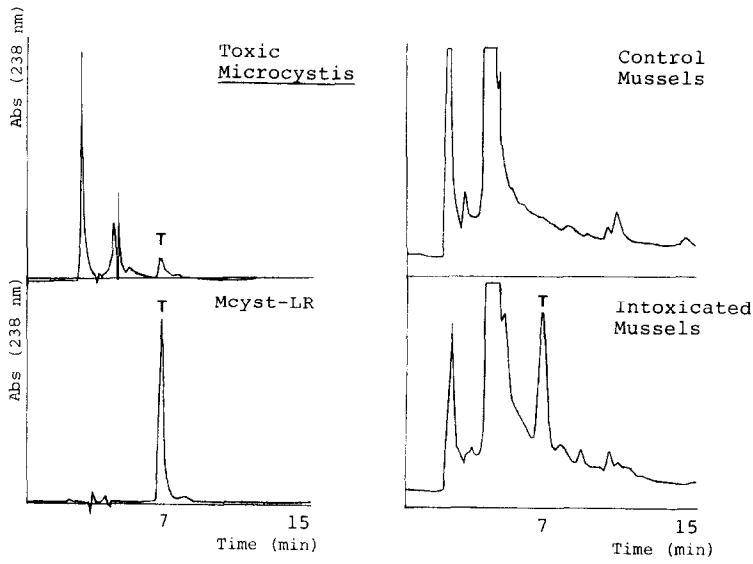

Fig. 2. HPLC chromatographs of MCYST-LR and of the extracts of the toxic *Microcystis*, control mussels and intoxicated mussels (T — toxin, conditions described in Material and methods).

2.2. *Depuration experiment*

Following day 16 of the uptake experiment, the mussels were fed daily for 15 days on a mixture of *Nitzschia* and *Tetraselmis* (1×10^5 cells/ml) with the water being changed as described. Every second day a sample of 20 mussels was taken and processed as previously described.

A control group of mussels was fed for the entire 31-day test period, only on the mixture of *Nitzschia* and *Tetraselmis* and another control group was starved during the period of the two experiments.

2.3. *Toxin analysis*

The freeze-dried homogenised cyanobacteria or mussels were extracted in a butanol/methanol/water (5:20:75) solution (50 ml solution/g dry homogenate). The method used is slightly modified from that of Eriksson et al. (1989). After extraction in a bath sonicator for 30 min the solutions were centrifuged (30 min, 6000 rpm) and the pellets reextracted. Toxin was partially purified using Bond Elut C18 columns and the toxic fraction was analysed on a reverse phase C18 HPLC column (Spherisorb S10 ODS2, 10×250 mm). Extracts were eluted with 0.01 M ammonium acetate/acetonitrile (26/74, v/v) on a Gilson HPLC system. The flow rate was 2 ml/min and the detector was set at 238 nm. Standard MCYST-LR (purity approx. 95%), kindly provided by Prof. Wayne Carmichael, was used as a standard. To confirm the pres-

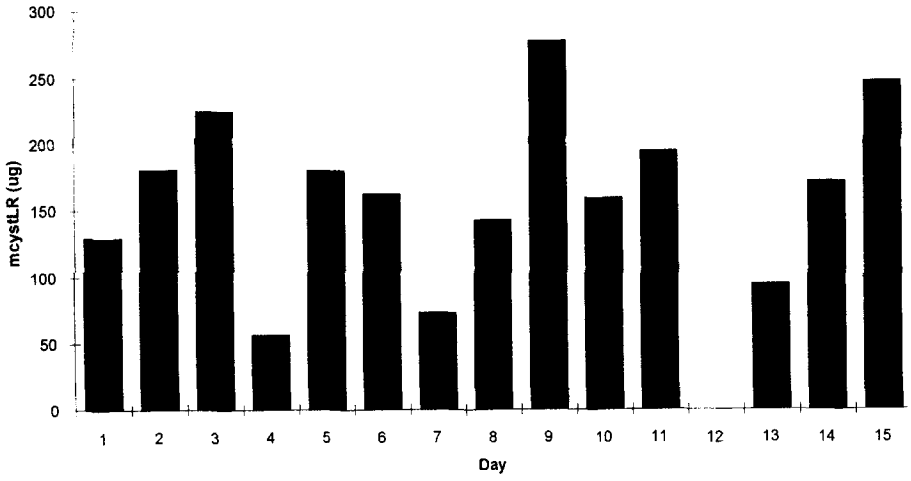

Fig. 3. Average daily amount of microcystin-LR (μg) provided to the mussels during the exposure experiment.

ence of the toxin, the peaks were analysed on a Hitachi 150–20 Spectrophotometer and the UV spectrum was compared with that of standard MCYST-LR.

3. Results

During the uptake experiment, mussel cumulative mortality was a low 0.5%, increasing gradually and slightly by the end of the depuration experiment to 6%. Both the control mussels that were unfed and those fed on marine algae had no mortality during the whole experiment. The animals showed production of pseudofaeces soon after being fed. During the whole experiment the mussels did not show any valve closure, bissus retraction or other stress symptoms.

The presence of MCYST-LR in the mussels is shown in Fig. 2. The chromatographs of the extracts of the toxic *Microcystis* cells, control mussels and intoxicated mussels are compared. It is clear that the toxin is taken up and a part is extractable. Dissected mussels had green contents in their guts confirming the ingestion of large amounts of *Microcystis*.

The amount of MCYST-LR provided to the mussels was not the same throughout the experiment (Fig. 3). The average cell content of MCYST-LR was 28.32 ± 13.67 $\mu g/10^8$ cells and the average daily amount given to the mussels was 153.00 ± 73.79 μg. This fluctuation of toxin content was due to the fact that several 4-l *Microcystis* cultures had to be used in order to have enough cell material for the daily feedings.

Small amounts of the toxin were detected in the mussels on the second day of the experiment, showing that toxin uptake was rapid. The uptake rate was fairly regular until day 10, decreased slightly and then remained stable over the following 6 days

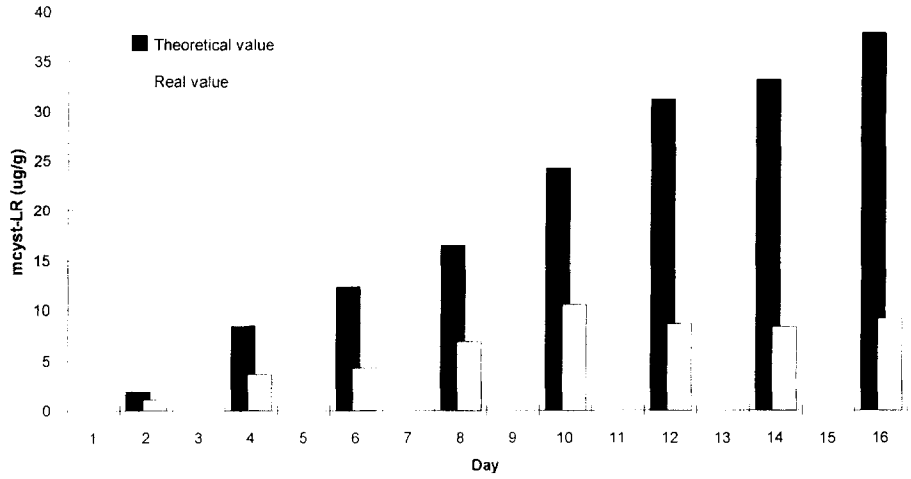

Fig. 4. Uptake of microcystin-LR (μg/g) in the mussels and maximum theoretical accumulation value during the 16-day experiment.

(Fig. 4). In Fig. 4 it can be seen that the amount of toxin taken up was only a part of the total provided. This percentage ranged from 54.8% on day 2 to 24.1% on day 16.

The maximum MCYST-LR taken up was 10.52 mg/g dry mussel weight at day 10.

After the animals were switched to a mixture of marine algae there was a 50% decrease until day 3 in the amount of toxin present in the mussels. From day 3 until day 7 there was a surprising increase in the amount of microcystin present in the tissues and after that it decreased and was not detectable at day 13 (Fig. 5). During this period pseudofaeces were also produced.

Distribution studies of the toxin in the mussel organs revealed that the major part

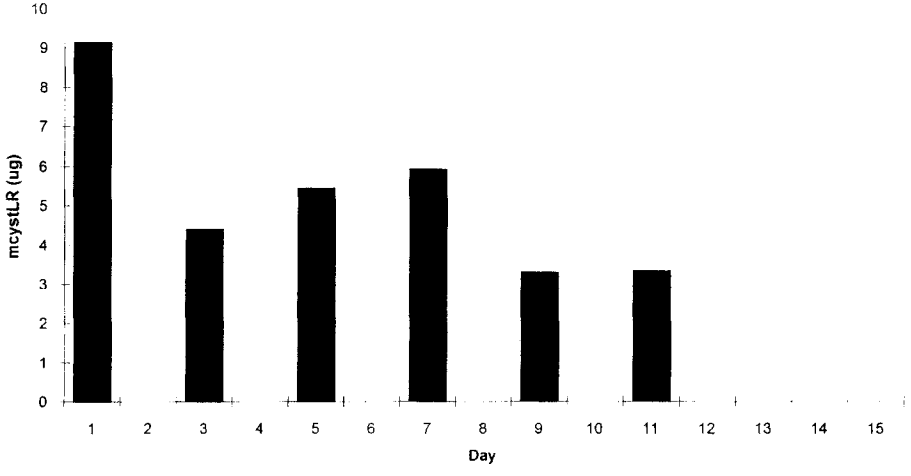

Fig. 5. Depuration of microcystin-LR (μg/g) from the mussels during the 15-day experiment.

of the toxin was present in the digestive tract, whereas the gills, foot, muscle and rest of the tissues had altogether less that 4% of the total toxin (Table 1).

4. Discussion

Mussel mortality was low during the uptake experiment, although it increased slightly during the depuration period. Falconer et al. (1992) did not report data on the mortality of *M. edulis* during their study on the occurrence of toxic blooms of *Nodularia*. On the other hand, Eriksson et al. (1989) state that *Anodonta* intoxicated with *Oscillatoria* did not seem to be affected by the toxins during a 12-day experiment. Nevertheless, nothing is reported on the survival of these molluscs after the experiment. Similar experiments done with molluscs and toxic dinoflagellates showed that mortality is dependent upon time following exposure (Lesser and Shumway, 1993), age of the bivalves, as well as the bivalves' prior exposure history. Shumway and Cucci (1987) reported that bivalve populations from areas prone to red tide outbreaks may have acquired resistance to the presence of toxic cells. Other parameters showing physiological changes such as oxygen uptake, present the same pattern, revealing that short-term exposures do not significantly affect these mechanisms (Mardsen and Shumway, 1993).

Behaviour of the animals during the uptake experiment was fairly normal. The molluscs rapidly filtered the cyanobacteria and produced pseudofaeces 10 to 20 min after being fed. Bricelj et al. (1990) point out that the production of pseudofaeces usually occurs at cell densities higher than 4×10^4 cells/ml along with a decrease in the filtration rates.

The amount of toxin in a cell depends on environmental factors such as nutrients, light and temperature (Van der Westhuizen and Eloff, 1985; Watanabe and Oishi, 1985; Sivonen, 1990), so toxin content in a cell is supposed to vary during a batch culture. That was probably responsible for the changes in toxin content of the *Microcystis* cultures used in this experiment.

Fluctuations of MCYST-LR taken up by the mussels during the experiment do not closely follow the theoretical concentrations calculated on the basis of the daily amount of MCYST-LR provided, divided by the number of mussels present on each day (Fig. 4). These values represent the maximum toxin possibly found in the mussels.

Table 1
Dry weight of the different mussel organs as a percentage of total body weight, MCYST-LR taken up and as percentage of toxin present in the different organs

Tissue	Dry weight (%)	MCYST-LR (μg/g)	Toxin (%)
Foot	3.8	0.00	0.0
Gills	21.9	0.29	1.0
Muscle	17.4	0.23	1.0
Digestive tract	16.0	27.60	96.5
Rest	40.9	0.46	1.5

Nevertheless, it should be noted that not all of the MCYST-LR provided is taken up because part of it may be rejected as faeces or pseudofaeces, while another portion may be irreversibly bound to protein phosphatases or metabolized. Considering the high density of *Microcystis* cells provided to the mussels, a decrease in the filtration activity resulting in lower amounts of MCYST-LR ingested should be also expected. The amount of MCYST-LR measured is the part not firmly bound to protein phosphatases or the amount that is present in the digestive tract of the mussels. MacKintosh et al. (1990) revealed that MCYST-LR is a potent specific inhibitor of protein phosphatases 1 (PP1) and 2A (PP2A) that are found in eucaryotic cells. They also stated that the interaction between MCYST-LR and PP1 and PP2A is very strong. Honkanen et al. (1990) suggested also that there is a strong interaction between MCYST-LR and the catalytic subunit of PP2A. These facts represent a problem when we are measuring MCYST-LR concentration extracted from animal tissues.

The decrease in the concentration of MCYST-LR in the mussel tissues after day 10 may be related to the fact that the amount of toxin provided after that period was much less than that given the day before. Likewise the slight increase on the last day of this experiment probably resulted from the higher MCYST-LR dose given the day before. Mussels seem to respond quite quickly to changes in toxin concentration in the *Microcystis* cells.

The decrease in the percentage of the toxin accumulated relative to that provided with time is not well understood. Part of the reason may be due to the fact that this theoretical value does not take into account the toxin lost in the pseudofaeces and faeces that are regularly produced by the mussels. This percentage increased a bit at day 10 when the amount of MCYST-LR in the same amount of cells was higher than the average. If the mussels filtered the same amount of cells as on other days they would ingest more toxin. It seems that there is a saturation point close to 11 μg/g, under the conditions of this assay. Lindholm et al. (1989) showed that *Anodonta* exposed to *Oscillatoria*-rich surface water from Lake Östra accumulated 30 μg of toxin/g dry weight mussel after 1 week. Eriksson et al. (1989) presented higher values for the accumulation of hepatotoxin from *Oscillatoria* in *Anodonta*. Nevertheless, one should take into account that these freshwater mussels are bigger and the feeding system was different.

In the experiment reported on here our goal was to mimic the situation that occurs in estuaries subjected to tides. In this case, the mussels may be exposed to high densities of cyanobacteria during short time periods each day, when high tides reach the mussel beds. The toxin levels attained in our experiment are similar to those obtained by Marcaillor-Le Baut et al. (1993) using *M. edulis* exposed to natural populations of *Dinophysis* spp., producers of okadaic acid.

During the depuration period it is interesting to note the fluctuation of toxin content in the mussels with time. The 50% decrease in toxin content on the second day after feeding with marine microalgae seems to be related to the fact that the mussels may have emptied the digestive tracts of their *Microcystis* contents. If so, the rest of the toxin was present in the mussel tissues not bound to protein phosphatases, and therefore more easily extractable. During the following days there was a surprising

increase in the toxin content of the mussels. No MCYST-LR was present in the water or in cells because the water was changed every second day. One possible explanation is that during this depuration period some of the MCYST-LR that was bound to protein phosphatases was liberated and released by the mussels.

After day 7 the toxin content in the mussel tissues decreased until no detectable amounts were found at day 13. The same pattern was found by Jamel Al-Layl et al. (1988) using *A. cygnea* and a strain of *Anabaena flos-aquae* producing neurotoxins and hepatotoxins simultaneously. It is not reasonable to expect that during these 11 days the toxin was present in *Microcystis* cells only in the digestive tract. It would be expected to be taken up in the mussel tissues and then released or metabolised by them.

The degradation of cyanobacteria toxins by eucaryotic organisms is not yet understood but it is known that these toxins can be degraded by chemical or biological mechanisms. Kiviranta et al. (1991) isolated a strain of the bacterium *Pseudomonas* that degrades anatoxin-a. Tsuji et al. (1994) revealed that MCYST-LR decomposed very slowly under exposure to sunlight alone but the addition of cyanobacteria pigments accelerated toxin decomposition and isomerization. The decomposition and isomerization rates were dependent upon the pigment concentration.

The distribution of the toxin in the different mussel tissues is similar to that observed when bivalves accumulate dinoflagellate toxins. Marcaillor-Le Baut et al. (1993) found that okadaic acid is primarily accumulated in the digestive gland. Bricelj et al. (1990) note that 96% of the PSP toxin from *Alexandrium fundyense* is accumulated in the organs of the digestive tract.

Results presented here show that there is a possibility of toxin uptake by mussels that live in estuaries where cyanobacteria blooms occur. Results presented are thought to represent a natural situation that would occur in our estuaries contaminated with toxic cyanobacteria water blooms. In some locations, however, the situation presented by our experiment may not represent the natural situation.

Nevertheless, human health may be affected when toxic blooms occur nearby mussels beds. Carmichael and Falconer (1993) recommend that 1 μg of cyanobacterial hepatotoxin per liter of drinking water should be regarded as the upper limit for safe consumption. This value was based on toxicity calculations from mouse dosing (Falconer et al., 1988) and from subchronic oral toxicity trials carried out on pigs (Falconer et al., 1994). Although these values are to be used in chronic situations, it is understood that they can be easily reached during a meal of intoxicated mussels containing the microcystin doses presented in this paper. A meal of mussels with levels of microcystin-LR such as those presented in this paper may represent an ingestion of around 250 μg of toxin per person, considering an ingestion of 625 g (total wet weight) of mussels per person containing 10.5 μg MCYST-LR/g dry mussel weight. One should consider that in situations where the uptake of microcystins is not affected by low filtration rates or by the production of pseudofaeces, these values may be tenfold higher. It is therefore recommended that the consumption of mussels during cyanobacteria blooms is to be avoided and a period of depuration should be established based upon the amount of toxins taken up by the mussels.

The behaviour of other bivalves such as clams and oysters may be similar but their

situation may be aggravated due to the fact that they are usually submerged during low tides. They are thus able to filter higher amounts of toxic cyanobacteria and accumulate more toxin. Further work is in progress in order to better estimate the accumulation and depuration mechanisms for microcystins under laboratory and field conditions.

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