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Accumulation and depuration of microcystins (cyanobacteria hepatotoxins) in *Tilapia rendalli* (Cichlidae) under laboratory conditions

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Abstract

In order to understand accumulation and depuration of microcystins (MCYSTs) in *Tilapia rendalli*, three experiments with juveniles were done. The experiments simulated the fish diet during a *Microcystis aeruginosa* bloom in three different situations. In the first one each fish received daily, during 15 days, fish food plus toxic cells of *M. aeruginosa* (20.4 μg MCYSTs fish^{-1} day^{-1}). In the following 15 days they were fed without toxic cells. In the second experiment, fish were fed only with toxic cells during 28 days (14.6 μg MCYSTs fish^{-1} day^{-1}) and in the third experiment, during 42 days, fish were fed with fish food plus toxic cells (29.2 μg MCYSTs fish^{-1} day^{-1}) previously disrupted (to simulate a senescent bloom). MCYSTs analyses were done by enzyme-linked immunosorbent assay (ELISA) in liver and muscle samples in all experiments and in faeces in the first one (only in the depuration period). The results demonstrated different profiles of MCYSTs accumulation in liver and muscle of *T. rendalli*. Comparing the experiments, the highest MCYSTs accumulation in the liver (2.8 μg g^{-1}) occurred in the second one, where fish had only toxic cells as feeding source. In the first experiment, the highest MCYSTs accumulation in liver (0.6 μg MCYSTs g^{-1}) was observed during the accumulation period, while in muscle, interestingly, the highest concentration (0.05 μg MCYSTs g^{-1}) occurred in the depuration period. In this same period, it was also observed elimination of toxins through faeces. The second and third experiments showed almost the same average concentrations in tissues although fish have received more MCYSTs in third one. With respect to implications of the fish comsumption, MCYSTs accumulation in muscle of *T. rendalli* in all three experiments reached concentrations that would represent an intake of these toxins above the tolerable limit for humans and these results confirmed our previous observations from a field study. In conclusion, in this study it was observed that *T. rendalli* is able to accumulate MCYSTs and the availability of other feeding sources, besides toxic cells, probably interferes with the accumulation rate. Therefore, the occurrence of toxic cyanobacterial blooms producing MCYSTs in aquaculture ponds could represent a risk to the quality of fish to the consumers.

Keywords: Microcystins; *Tilapia rendalli*; Accumulation; Depuration

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1. Introduction

Eutrophic freshwater ecosystems favour the intense development of cyanobateria blooms. These microorganisms are potentially toxins producers able to damage aquatic biota and mammals (Yoo et al., 1995). In Brazil, the increasing of eutrophication in some lakes, lagoons and reservoirs have allowed the frequent dominance of cyanobacteria in phytoplankton (Huszar et al., 1998). Moreover, according to our previous studies (unpublished data) the majority (82%) of cyanobacteria strains isolated from Brazilian freshwater ecosystems showed to be toxin producers when tested by bioassays, immunoassays or by HPLC analyses.

There are few ecological studies investigating the environmental role of these toxins. Even though existing a great number of herbivorous and phytoplanktivorous fish species, there is no much knowledge about effects of toxic cyanobacteria on fish and its responses to cyanotoxins, mainly in sub-lethal or chronic exposure situations.

Cyanobacteria are possibly an important component of tropical cichlids and cyprinids diet (Bowen, 1982 in Beveridge et al., 1993). However, toxins can limit fish feeding on cyanobacteria. Beveridge et al. (1993) showed a supression in filtration rate and growth of two tilapia species, *Hipophthalmichthys molitrix* and *Oreochromis niloticus*, in presence of toxic *Microcystis aeruginosa*. Nevertheless, many fish species are not able to avoid ingestion of these toxic organisms in environments such as small lakes and aquaculture ponds (Tencalla and Dietrich, 1997).

Microcystins (MCYSTs) are the main group of cyanotoxins already studied. These molecules are hepatotoxic cyclic heptapeptides, which present more than 60 variables generally differing in the nature of the two L-amino acids and in the degree of methyl substitution. These cyanotoxins are potent and specific inhibitors of protein phosphatases (serine/threonine family), especially PP1 and PP2A (Chorus and Bartram, 1999). Some studies on acute effects of MCYSTs in fish evaluated the inhibition of PP1 and 2A in liver. Sahin et al. (1995) fed trouts with *M. aeruginosa* extracts containing MCYSTs and showed 50% inhibition of protein phosphatases activity with 0.05 nM of toxin. Tencalla and Dietrich (1997) observed complete inhibition of proteins phosphatase with 2 μg of MCYST-LR g^{-1} in the liver of trouts.

In acute toxicity, this enzymatic inhibition can result in morphologic damage in the liver. Andersen et al. (1993) exposed the Atlantic salmon to high doses of MCYST-LR and observed that this toxin caused diffuse necrosis and megalocytosis in the whole liver. That experiment reproduced exactly the symptoms found in salmons of North America with the "netpen liver disease" which caused serious economic losses to that region. Fish mortality caused by MCYSTs was also observed experimentally by Tencalla et al. (1994). These authors verified that trouts gavaged with *M. aeruginosa* freeze-dried cells (dose of 6.6 mg MCYST-LR kg^{-1} body weight) died in a 96-h period. Nodularin, another hepatotoxin produced by cyanobacteria with effects similar to MCYSTs, was also found producing severe damages to liver of sea trouts (Kankaanpää et al., 2002).

Bioaccumulation evidences were obtained from salmons that ate crab larvae containing MCYSTs (Williams et al., 1997). However, some fish are able to depurate MCYSTs accumulated in the liver through biliary excretion (Sahin et al., 1996). As occurs in mammals, the process of MCYSTs detoxication in aquactic organisms is related to a conjugation reaction of MCYSTs to glutathione through the activity of glutathione *S*-tranferase (Pflugmacher et al., 1998). Excretion and detoxication are ecologically important, considering that it could limit the bioaccumulation of MCYSTs in the trophic chain.

In Brazil, introduction of tilapia species such as *Tilapia rendalli* and *O. niloticus*, for socioeconomic purposes, has been done since 1956 (Gurgel and Fernando, 1994). However, the consequent nutrient enrichment of these aquatic ecosystems caused by such activities has led to frequent cyanobacterial blooms (Starling, 1993).

Jacarepaguá lagoon, a brackish shallow lagoon located at Rio de Janeiro city (23°00′ lat, 43°20′ long) is a typical example of aquatic ecosystem affected by anthropic impacts. This lagoon presents an increasing eutrophication and heavy blooms of cyanobacteria, mainly toxic *M. aeruginosa*. A decrease in the fish production has also been observed during the last 10 years. At present, the main fish species in that lagoon is *T. rendalli* and our previous field study showed high MCYSTs concentration in liver of this fish, which also presented hemorrhagic symptoms. These cyanotoxins

were found in muscle and viscera as well. (Magalhães et al., 2001).

In this paper, we report laboratory experiments done with *T. rendalli*, under different conditions of toxic cells ingestion, in order to observe MCYSTs accumulation and depuration in this species. The risk estimation for human consumption of this fish species containing MCYSTs is also discussed.

2. Material and methods

2.1. M. aeruginosa culture

The toxic *M. aeruginosa* strain (NPLJ-4) was isolated from Jacarepaguá Lagoon (Rio de Janeiro, Brazil). This strain was cultured in ASM-1 medium (Gorham et al., 1964), pH = 8.0, temperature of 23 ± 2 °C, 22 μE m^{-2} s^{-1} of light intensity and photoperiod of 12 h. In the end of exponencial growth phase, cells were concentrated through a tangencial flow multiple filter technique using the Pellicon Cassettt System (Millipore).

2.2. Quantitation of MCYSTs produced by NPLJ-4 strain

Previous NMR analysis of the NPLJ-4 extract showed presence of four microcystins congeners. MCYST-LR represented 80% of the total and the other three congeners could not be characterized due to very low concentration for NMR analyses. For this study, NPLJ-4 extract was analyzed by HPLC and the sum of the four microcystin peak areas was applied to a MCYST-LR standard curve to calculate the MCYST-LR equivalents present in the extract and produced per cell.

Briefly, NPLJ-4 cells concetrate was extracted three times at room temperature with a butanol:methanol:water (5:20:75 v/v) solution and analyzed following the procedure described by Krishnamurthy et al. (1986) and Azevedo et al. (1994). The extract was partially purified in C18 cartridge (500 mg Bond-Elut, Varian) and eluted with 100% methanol. This fraction containing the toxins was dried and resuspended in deionized water and MCYSTs analysis was performed by PDA-HPLC (Shimadzu SPD-M10A) using a Lichrospher 100 RP-18 reverse phase analytic column (5 μm, Merck). The chromatography was carried out under isocratic conditions with a mobile phase of acetonitrile and 20 mM ammonium acetate pH = 5.0 (28:72 v/v), for 10 min. The injected volume was 20 μL with a flow rate of 1 mL min^{-1}. The UV detection was done at 238 nm and the absorption spectrum of each peak was analyzed over the range of 190–300 nm. The quantitation of MCYSTs was done using a MCYST-LR standard curve (standard purchased from Sigma–Aldrich). This analysis allowed us to detect a production of 7.8×10^{-3} ng of MCYST-LR equivalents per cell. Such value was used to calculate the amount of toxin provided to each fish in the experiments.

2.3. T. rendalli capture and maintenance

Fish were obtained from an aquaculture pond free of toxic cyanobacteria. Only juveniles (sexually immature) with 5 ± 1 cm length were collected. The fish were held in aquariums with 60 L of dechlorined water. The aquariums were also set up with a continuous system of water filtration and aeration and the temperature was kept constant (23.5 ± 0.5 °C). Fish were fed with commercial fish food and were acclimatized during 2 weeks before the beginning of the experiments.

2.4. Experiments

The best method found to provide *M. aeruginosa* cells to fish was a food composed by the mixture of NPLJ-4 cells concentrate plus 2% agar (including fish food or not, depending on each experiment), which gave the food a consistency of jelly and allowed to have better control on the amount of food ingested by fish. The appropriate amount of food verified for them was 1 g per day per fish. The calculation of MCYSTs offered to fish in each experiment was based on estimation that each tilapia could filter around 1 l of water per day. Considering a cyanobacteria heavy bloom situation in Jacarepaguá Lagoon, each fish would ingest around 10^8 cells per day (Table 1).

The first experiment was carried out using three aquariums (one control and two test aquariums), with 20 fish in each one. It was performed with two different phases: in the first one, each fish in test aquariums

Table 1
Experimental conditions

Experiment number	Duration (days)	Phase	Number of fish (total)	Food (1 g per fish)	MCYSTs ($\mu g\,g^{-1}$)	Cells (cells g^{-1})	Fish collected in each interval
		(1) Accumulation 15 days	30	C[a]-agar + fish food T[b]-agar + fish food + toxic *M.a.*	20.4	2.6×10^8	2 fishes per aquarium per 3 days
1	30	(2) Depuration	30	C-agar + fish food			2 fishes per aquarium per 3 days
		15 days		T-agar + fish food C-agar + fish food			Faeces per 3 days
2	28	Accumulation	36	T-agar + toxic *M.a.* C-agar + fish food	14.6	1.9×10^8	3 fish per aquarium per 7 days
3	42	Accumulation	54	T-agar + toxic *M.a.*	29.2	3.8×10^8	3 fish per aquarium per 7 days

M.a.: Microcystis aeruginosa.
[a] C: control.
[b] T: test.

received during 15 days the jelly food containing commercial fish food plus toxic cells of *M. aeruginosa* (20.4 μg MCYSTs $fish^{-1}$ day^{-1}). In the following 15 days these fish were fed with the same food without toxic cells as in the control aquarium. Two fish from each aquarium were collected every 3 days, totalizing 10 fish per aquarium at the end of each phase. Faeces were collected only during the second phase for MCYSTs analyses.

In the second experiment, fish in test aquariums were fed with the jelly food containing only toxic cells of *M. aeruginosa* (14.6 μg MCYSTs $fish^{-1}$ day^{-1}) during 28 days.

In the third experiment, fish in test aquariums were fed with the jelly food containing commercial fish food plus toxic cells of *M. aeruginosa* previously disrupted (29.2 μg MCYSTs $fish^{-1}$ day^{-1}), during 42 days. The volume of NPLJ-4 cells concentrate used to prepare food was previously subjected to three cycles of freeze–thawing to disrupt the cells. In these two last experiments, three fish from each aquarium were collected every 7 days (Table 1).

The aim of the experiments was to represent three different ecological situations. The first one would simulate the fish diet during and after a toxic *M. aeruginosa* bloom, existing all the time other feeding sources (represented in the experiment by the fish food). The second one would simulate the fish eating cells from a toxic bloom, not having any other feeding source. Finally, the third experiment would represent fish feeding on toxic and disrupted cells (dissolved MCYSTs) from a senescent bloom, also having other feeding sources.

2.5. Extraction and analyses of MCYSTs from fish tissues and faeces

For the analyses, all fish collected from each test aquarium in the same interval of time (Table 1) had their tissues removed (liver or muscle) and pooled, forming one sample of liver and one sample of muscle. These samples were weighed and extracted twice with 100% methanol. Methanol extract was mixed three times with equal volume of hexane. Hexane layers were discarded; methanol extract was dried in glass beckers and resuspended in deionized water. This extract was loaded onto a C18 cartridge, which was washed with 30 mL of deionized water, followed by 30 mL of 20% methanol, and finally eluted with 50 mL of 100% methanol. This last fraction was dried in glass beckers and resuspended in 1.0 mL of deionized water, stored in eppendorf vial in freezer at −20 °C for a short period of time and this volume was analyzed by ELISA method using microcystin plate kits (Envirologix Inc.®). Each sample was analyzed with replica and MCYSTs values were expressed as MCYST-LR equivalents (Magalhães et al., 2001).

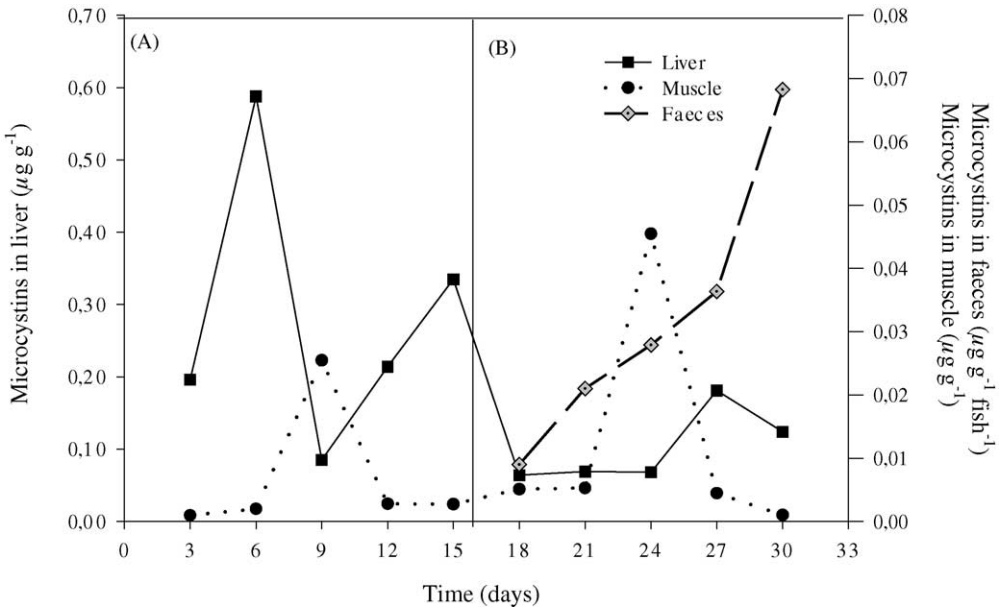

Fig. 1. Microcystins concentration in liver and muscle of *T. rendalli* during the accumulation (A) and depuration (B) periods in the first experiment. In the depuration period is also presented the microcystins concentration in faeces. In this experiment the jelly food contained commercial fish food plus toxic *M. aeruginosa* cells (MCYSTs offered to fish = 20.4 μg (fish day^{-1}).

Faeces were collected with a glass pipette from the bottom of each aquarium in the first experiment (Table 1). It was dried at 50 °C, weighted and extracted twice by the same methodology described for toxin extraction from *M. aeruginosa* cells in Section 2.2. MCYSTs concentration in the faeces material was also determined using ELISA plate kits.

The extraction procedures and the ELISA method used to analyze the samples are not suitable to detect MCYST bound to glutathione or protein phosphatases, so all results in this study refer to free MCYSTs in the fish tissues.

3. Results

MCYSTs accumulation in liver and muscle of *T. rendalli* during the first experiment can be observed in Fig. 1. The highest concentration in liver was observed on the sixth day, when it was detected 0.6 μg MCYSTs g^{-1}. The average concentration in the accumulation period was 0.28 μg MCYSTs g^{-1} (±0.19S.D.). From the 15th day on (depuration period) MCYSTs concentration in liver decreased and the average concentration was 0.10 μg MCYSTs g^{-1} (± 0.05S.D.), about three times less than in the accumulation period.

MCYSTs concentration in muscle was much lower than in liver, certainly because liver is the target organ of these toxins. The highest concentration in muscle during the accumulation period (0.03 μg MCYSTs g^{-1}) was observed on the nineth day and the average concentration was 0.007 μg MCYSTs g^{-1} (±0.01S.D.). In the depuration period, the highest concentration (0.05 μg MCYSTs g^{-1}) was observed on the 24th day (Fig. 1) and the average concentration of 0.012 μg MCYSTs g^{-1} (±0.02S.D.) was higher than in the accumulation period.

MCYSTs concentration in faeces increased linearly until the end of the depuration period (Fig. 1). The highest concentration observed on the last day was 0.07 μg MCYSTs g^{-1} faeces per fish and the average concentration in this period was 0.03 μg MCYSTs g^{-1} faeces per fish (±0.02S.D.). The sum of MCYSTs concentrations detected in faeces was 0.16 μg MCYSTs g^{-1} faeces per fish. Thus, in the end of this experiment we could estimate that fish excreted in the depuration period approximately 0.06% of the estimated total

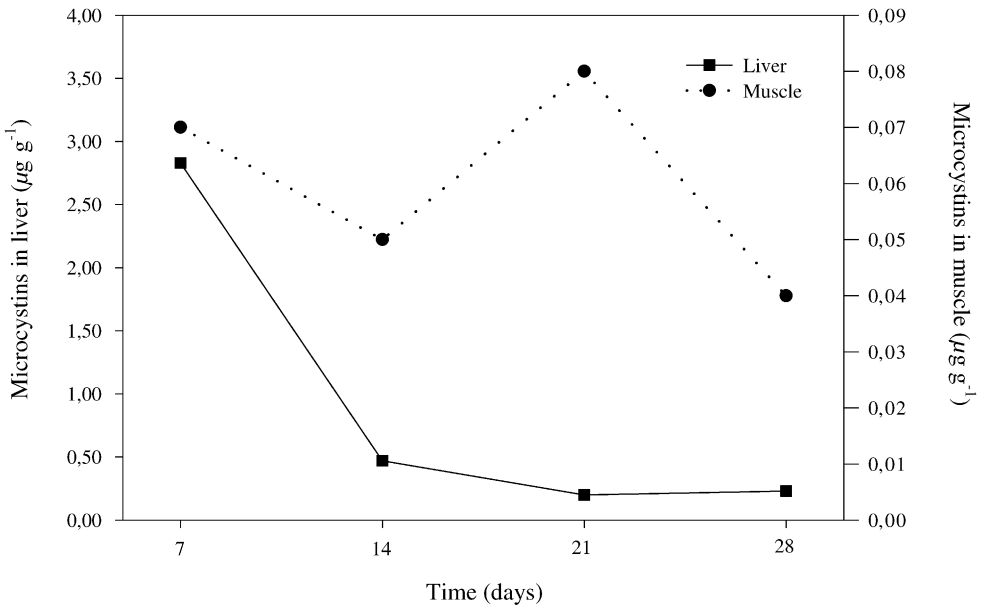

Fig. 2. Microcystins concentration in liver and muscle of *T. rendalli* during the second experiment which the jelly food contained only toxic *M. aeruginosa* cells (MCYSTs offered to fish = 14.6 μg (fish day^{-1})).

amount of MCYSTs ingested in the accumulation period.

In the second experiment it was observed that the highest MCYSTs concentration in liver (2.8 μg g^{-1}) occurred in the first sampling date (Fig. 2). In muscle, the highest MCYSTs concentration (0.08 μg g^{-1}) was observed in third week.

In the third experiment (Fig. 3), differently from the other ones, the highest MCYSTs concentration in liver (1.7 μg g^{-1}) occurred in the sixth week and in muscle the highest concentration (0.1 μg g^{-1}) was observed in the second week. However, comparing the average concentrations, these two last experiments showed almost the same values. Liver average concentration was 0.93 μg g^{-1} (±1.2S.D.) in the second experiment and 0.92 μg g^{-1} (±0.5S.D.) in the third experiment. Muscle average concentration was 0.06 μg g^{-1} (±0.018S.D.) in the second experiment and 0.05 μg g^{-1} (±0.028S.D.) in the third one.

The estimated daily intake of MCYSTs for an adult weighing 60 kg ingesting 300 g of muscle of *T. rendalli* growing in the same conditions of each experiment is shown in Fig. 4. In the first experiment, the averages of estimated values of MCYSTs ingestion were 0.03 μg kg^{-1} body weight day^{-1} (±0.05S.D.) in the accumulation period and 0.06 μg kg^{-1} body weight day^{-1} (±0.09S.D.) during the depuration period. The WHO suggests as the tolerable daily intake (TDI) of MCYSTs, 0.04 μg kg^{-1} body weigh day^{-1} (Chorus and Bartram, 1999), which means that during the depuration period the daily intake could be higher than the safety value. In the first experiment there were samples presenting values of MCYSTs in the muscle that would represent an intake up to six times higher than suggested by the WHO (Fig. 4).

In the other conditions (second and third experiments), the consumption of those fish would represent an estimated ingestion of MCYSTs above the tolerable limit for humans during all period (average of 0.29 ± 0.09 and 0.24 ± 0.12 μg kg^{-1} body weight day^{-1} for second and third experiments, respectively). On the 14th day of the third experiment the MCYSTs concentration was 12 times above the limit (Fig. 4).

4. Discussion

In the first experiment the highest MCYSTs concentration in liver of *T. rendalli* occurred in the accumulation period followed by a clear decrease in the

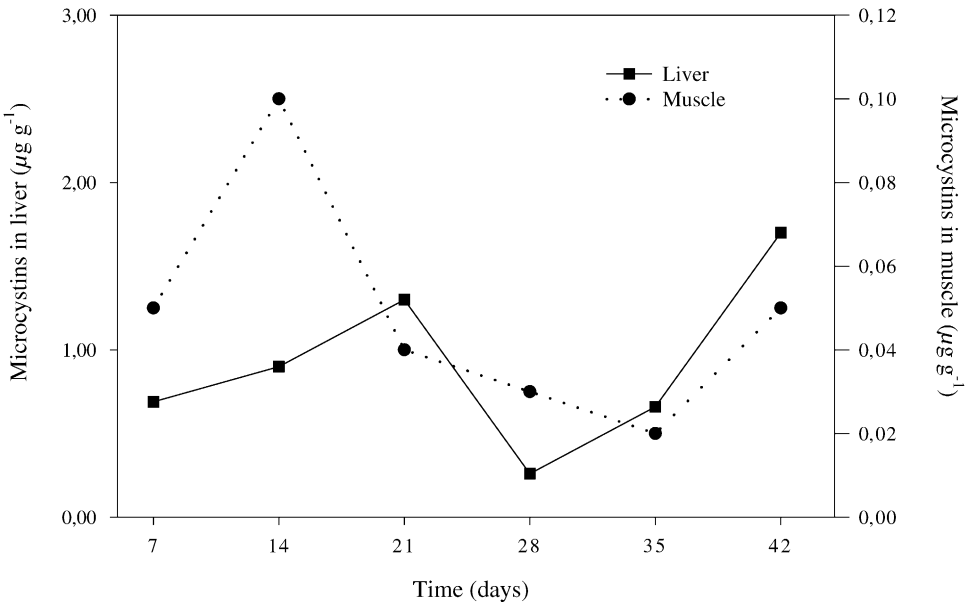

Fig. 3. Microcystins concentration in liver and muscle of *T. rendalli* during the third experiment which the jelly food contained commercial fish food plus lysed toxic *M. aeruginosa* cells (MCYSTs offered to fish = 29.2 μg (fish day^{-1})).

depuration period. Interestingly, in the muscle it could be observed an increase in MCYSTs concentration during the depuration period. These results suggest that, in the nature, the toxins could still be found in the fish muscle several days after the end of a toxic bloom. It is important to emphasize that MCYSTs concentration detected in the fish tissues refers to free toxins, once it was not possible to detect MCYSTs bound to protein phosphatases or glutathione using the available method applied to this work. Consequently, total microcystins (bound plus unbound) in those tissues are underestimated. Vasconcelos (1995) and Amorim and Vasconcelos (1999) also observed higher MCYSTs concentrations in depuration periods in experiments with mussels. According to these authors this fact can be a consequence of the metabolization of proteins phosphatases, a turnover that would lead to releasing of these toxins, allowing its detection by the ELISA method.

The excretion of MCYSTs through faeces verified in the first experiment has already been reported in the literature and can be due to bile excretion, considered as the main excretion via for these toxins (Falconer and Humpage, 1996, Råbergh et al., 1991; Sahin et al., 1996). Amorim and Vasconcelos (1999) also observed that faeces of mussels still presented MCYSTs in the 14th day of the depuration period. It is possible that most of the MCYSTs present in faeces was bound to glutathione or to a final product of the detoxication via. However, the clean-up steps of the extration procedure probably removed the conjugates from the samples. Considering this, it is expected that the real MCYSTs concentration in the fish faeces is higher.

In the second and third experiments the highest MCYSTs concentrations in the tissues occurred in different sampling dates. Its important to emphasize that in the second experiment MCYSTs concentration offered to fish was a half part of the amount offered in the third experiment and, in despite of this, it could be observed the same average concentration in the tissues in both experiments. The difference between these two experiments was that in the second one fish were fed only with toxic cells and in the third one the toxic cells offered to the fish were previously disrupted and complemented with commercial fish food. These results indicate that when fish feed only on cyanobacteria cells, a possible situation when a bloom occurs, MCYSTs uptake can be higher than when fish have other feeding source and toxins are more available as in the third experiment.

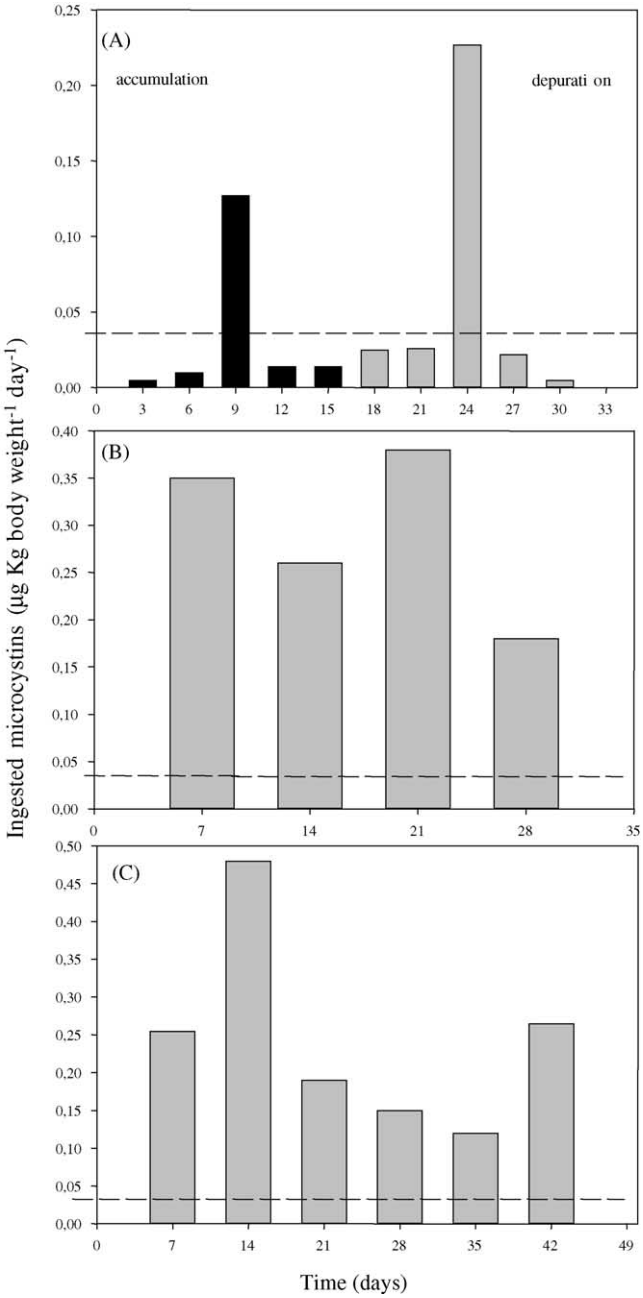

Fig. 4. Estimated intake of microcystins by a person consuming 300 g of *Tilapia rendalli* in each experimental condition ((A) first experiment, (B) second experiment, (C) third experiment). The horizontal line indicates the maximum tolerable daily intake for humans (0.04 $\mu g\,kg^{-1}$ body weight day^{-1}) proposed by the WHO (Chorus and Bartram, 1999).

The results obtained with this study demonstrated that MCYSTs accumulation in liver and muscle of *T. rendalli* occurred with different profiles. Comparing the three experiments, it seems that the situation where fish had only toxic cells of *M. aeruginosa* as feeding source, as represented by the second experiment, would be the situation of highest MCYSTs accumulation.

Decreases of MCYSTs concentration in the tissues might also be associated to excretion of MCYSTs through the faeces. This toxins elimination was verified during the depuration period in the first experiment and might has also occurred in the accumulation period and during the other two experiments. Although the low percentage of excreted MCYSTs during the depuration has not been relevant in relation to the estimated total amount of MCYSTs ingested in the accumulation period, these percentages might be understimated due to the impossibility to detect MCYSTs conjugates originated from detoxication process.

We verified experimentally MCYSTs accumulation in muscle of *T. rendalli*, a fish species frequently found in several Brazilian fish pounds and reservoirs presenting heavy *Microcystis* bloom. In fact, in our previous study, MCYSTs have been detected in muscle of fish collected from a brackish lagoon (Magalhães et al., 2001). In this environment, *Microcystis* cells density in the water commonly allows fish to ingest a great number of cells, equivalent to cells density used in our experiments. In another field study, MCYSTs were also found in muscle of *Oreochromis niloticus* obtained from a fish farm (Mohamed et al., 2003). All those studies verified MCYSTs levels in muscle that would not be recommended for human consumption.

M. aeruginosa blooms have also occurred frequently in aquatic ecosystems used for aquaculture and fishery due to the consequent eutrophication caused by such economic activities. Therefore, commercialization of fish from these environments could represent a risk for public health. Our study showed that consumption of muscle of *T. rendalli* growing in similar conditions of the three experiments would lead to an intake of MCYSTs above the tolerable limit for humans. It is important to observe that the proposed limit by the WHO of 0.04 μg MCYSTs kg^{-1} body weigh day^{-1} was estimated for adults. Hence, the risk for children needs to be more carefully considered.

5. Conclusion

In this study it was observed that *T. rendalli* is able to accumulate MCYSTs and the availability of other feeding sources besides toxic cells probably interferes with the accumulation rate.

Chronic exposure to MCYSTs, characterized by its oral consumption through fish during a long period, can represent a risk to human health, once these toxins are hepatic tumor promoters (Falconer and Humpage, 1996). Therefore, the occurrence of toxic cyanobacterial blooms produncing MCYSTs in aquaculture ponds can represent a risk to the quality of fish to be consumed and consequently, this exposure route should become an important concern for the public health authorities.

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