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Uptake, tissue distribution and accumulation of microcystin-RR in *Corydoras paleatus*, *Jenynsia multidentata* and *Odontesthes bonariensis* A field and laboratory study

Jimena Cazenave[a,b], Daniel Alberto Wunderlin[b], María de los Ángeles Bistoni[a], María Valeria Amé[b], Eberhard Krause[c], Stephan Pflugmacher[d], Claudia Wiegand[d,e,*]

[a] *Universidad Nacional de Córdoba, Facultad de Ciencias Exactas, Físicas y Naturales, Cátedra Diversidad Animal II, Velez Sarsfield 299, 5000 Córdoba, Argentina*

[b] *Universidad Nacional de Córdoba, CONICET, Facultad de Ciencias Químicas, Dto. Bioquímica Clínica, CIBICI, Haya de la Torre esq. Medina Allende, Ciudad Universitaria, 5000 Córdoba, Argentina*

[c] *Institute of Molecular Pharmacology, Robert-Rössle Str. 10, 13125 Berlin, Germany*

[d] *Leibniz Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 301, 12587 Berlin, Germany*

[e] *Humboldt University at Berlin, Department of Biology, Unter den Linden 6, 10099 Berlin, Germany*

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Abstract

The uptake and accumulation of microcystin-RR (MC-RR) in fish was investigated under laboratory conditions and in wild fish. *Jenynsia multidentata* and *Corydoras paleatus* were exposed for 24 h to 50 μg/L MC-RR dissolved in water. After exposure, liver, gill, brain, intestine, gall bladder, blood and muscle were analyzed for MC-RR by HPLC and analysis confirmed by LC-ESI-TOF–MS spectrometry. Furthermore, wild individuals of *Odontesthes bonariensis* were sampled from the eutrophic, cyanobacteria-containing San Roque reservoir, and analyzed for the presence of MC-RR in liver, gill, intestine, and muscle.

MC-RR was found in liver, gills, and muscle of all exposed and wild fish, while in *C. paleatus* MC-RR was also present in the intestine. Moreover, we found presence of MC-RR in brain of *J. multidentata*. Results indicate that MC-RR uptake might occur at two different organs: intestine and gills, through either feeding (including drinking) or respiratory activities. This suggests that MC-RR is taken into the blood stream after absorption, and distributed to different tissues. The liver showed the major bioaccumulation of MC-RR in both experimentally exposed and wild individuals, with muscle of wild fish showing relative high amounts of this toxin in comparison with those exposed in the laboratory; though MC-RR was present in muscle of fish exposed for 24 h. The amount of MC-RR in muscle of *O. bonariensis* exceeded the value suggested by WHO to be safe, thus causing a health risk to persons consuming fish as a result of chronic exposure to microcystin. Gills also showed bioaccumulation of MC-RR, raising questions on the mechanism involved in the possible uptake of MC-RR through gills as well as on its accumulation

* Corresponding author. Tel.: +49 30 64181 639; fax: +49 30 64181 682.
E-mail address: cwiegand@igb-berlin.de (C. Wiegand).

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in this organ. Although MC-LR has been reported in brain of fish, this is the first report confirming the presence of MC-RR in this organ, which means that both toxins are able to cross the blood–brain barrier. These findings also raise questions on the probable neurotoxicity of microcystins.

Keywords: Cyanotoxin; Fish; Distribution; Brain

1. Introduction

Cyanobacteria produce a wide range of potent toxins (Carmichael, 1997). Globally, the most frequently found cyanotoxins in fresh and brackish water blooms are the cyclic peptide toxins of the family of microcystins (MC) and nodularins (Chorus and Bartram, 1999). Over 70 structural analogues of MC have been identified (Fastner et al., 2002). Most of the toxin is released into the water after lysis of cyanobacterial cells. The concentrations of dissolved toxin in the environment vary from traces up to 1800 μg/L or higher, immediately after the collapse of a highly toxic bloom (Chorus and Bartram, 1999). For this reason, either oral ingestion or immersion are the environmentally relevant routes of toxin exposure.

Toxic effects of microcystin-LR (MC-LR) on several organisms have been assessed in many investigations (e.g. Kotak et al., 1996; Zambrano and Canelo, 1996; Oberemm et al., 1997; Palíkóva et al., 1998; Pflugmacher et al., 1998; Vajková et al., 1998; Wiegand et al., 1999; Fischer et al., 2000; Best et al., 2002; Liu et al., 2002; Li et al., 2003; Malbrouck et al., 2003; Baganz et al., 2004; Jacket et al., 2004). Despite of their wide distribution and abundance in the environment, the effects of microcystin-RR (MC-RR) on aquatic organisms have been studied less extensively (Fischer and Dietrich, 2000a; Pietsch et al., 2001; Xie et al., 2004, 2005; Cazenave et al., 2005a,b).

Different exposure routes have been used to evaluate toxic effects of MC on fish. Some of them include intraperitoneal injection, oral ingestion (by natural or forced feeding = gavage), and bath immersion (MC dissolved in water) (Landsberg, 2002). It is well known that the toxicity depends on the uptake route. Thus, injected MC causes stronger effects than fed toxin. Exposure via immersion causes the lowest effects. For instance, Tencalla et al. (1994) showed that gastrointestinal uptake after forced feeding caused massive hepatic necrosis followed by fish deaths, whereas immersion of adults and juveniles in contaminated water did not produce toxic effects.

When MC is absorbed and distributed into an aquatic organism, it can be biotransformed. Biotransformation products can be transported to the kidneys and intestine for subsequent excretion (Pflugmacher et al., 1998). MC can also be accumulated in different body compartments of fish. The mechanisms of uptake and consequent distribution of MC in different tissues in fish are scarce. On the other hand, there are several reports on accumulation of MC in different organs of fish fed (naturally or by gavage) with either toxic cyanobacteria cells or injected intraperitoneally with radiolabeled MC. Thus, MC has been detected in liver, intestine, kidney, muscle, gallbladder and blood of fish experimentally exposed to the toxin (Williams et al., 1995; Sahin et al., 1996; Tencalla and Dietrich, 1997; Bury et al., 1998b; Malbrouck et al., 2003; Xie et al., 2004). Furthermore, some field studies have also detected MC in different fish tissues (Magalhaes et al., 2001, 2003; Sipiä et al., 2001; Mohamed et al., 2003; Xie et al., 2005). The uptake of MC from the medium has been demonstrated in zebra fish embryos exposed to dissolved ^{14}C-MC-LR (Wiegand et al., 1999). Immersion of fish in water containing MC caused alteration of behavior (Baganz et al., 1998, 2004) as well as reaction of enzymatic systems (Wiegand et al., 1999; Pietsch et al., 2001; Snyder et al., 2002). To our knowledge, there are no previous reports confirming the accumulation of water-dissolved MC in different fish tissues.

According to Ito et al. (2002), the toxicity of MC depends on the balance between accumulation and metabolism. In a recent work, we observed that the detoxification capacities of MC-RR via the glutathione-*S*-transferase (GST) pathway were stronger in *Jenynsia multidentata* than in *Corydoras paleatus*. Furthermore, GST of both fish species was inhibited at 20 and 2 μg/L dissolved MC-RR, respectively (Cazenave et al., 2005b). Thus, we hypothesize

that at the applied concentration of 50 μg/L accumulation could occur because of inhibition in the metabolic pathway leading to MC excretion.

The main goal of this work was to examine the uptake, accumulation and tissue distribution of MC-RR in fish experimentally exposed to dissolved toxin. Additionally, we aimed to verify results obtained in the laboratory study by evaluating the accumulation of MC-RR in wild fish exposed to toxic cyanobacterial blooms. Results show that MC-RR is taken into fish from both water and bloom material, both via the gills and intestine, followed by distribution and accumulation in different organs.

2. Materials and methods

2.1. Laboratory study

C. paleatus (Pisces, Callichthyidae) and *J. multidentata* (Pisces, Anablepidae) are native widespread neotropical fish species. They were selected due to their favorable experimental properties (abundance, small size, easy collection from rivers and streams, and unproblematic maintenance in aquarium). Specimens were collected by a backpack electrofisher (Coffelt, model Mark 10) from an unpolluted site (Suquia River at La Calera, Córdoba, Argentina) (Wunderlin et al., 2001; Hued and Bistoni, 2002) and transported to the laboratory.

Fish were acclimatized to controlled aquarium conditions (water reconstituted from deionized water with the following added salts: 100 mg/L sea salt, 200 mg/L $CaCl_2$, 103 mg/L $NaHCO_3$), temperature controlled at 21 ± 1 °C, light:dark cycle of 12 h:12 h during 4 weeks prior to experiments. Fish were fed once a day with commercial fish pellets, and starved 24 h prior to experiment.

Two groups of *C. paleatus* (five individuals each) and two groups of *J. multidentata* (five individuals each) were exposed to MC-RR at concentration of 50 μg/L, each one with its respective control group (five individuals each). The average weight of *C. paleatus* was 1.91 ± 0.34 g ($n = 20$), while the corresponding to *J. multidentata* was 1.53 ± 0.32 g ($n = 20$). After 24 h exposure, fish were washed with deionized water for 30 s (in order to remove MC-RR from their surfaces), and then sacrificed and dissected. Liver, gill, intestine, gallbladder, brain and muscle from control and exposed animals were separated, pooled, and stored at −20 °C until MC analysis. In the case of *C. paleatus*, also blood samples were obtained for MC analyses from the caudal vein by dissection of the caudal peduncle and collecting the blood using heparinized microtips. It was not possible to obtain blood samples from *J. multidentata*. The weight of each fresh tissue pool was recorded.

2.2. Field study

Odontesthes bonariensis (Pisces, Atherinidae) were captured from San Roque reservoir (Córdoba, Argentina; 31°21′S, 64°30′W), which has been classified as eutrophic to hypereutrophic with elevated concentrations of nutrients, and high incidence of toxic cyanobacterial blooms (Amé et al., 2003). Water samples, bloom material and fish were collected during 2004. Samplings were performed once during the wet season and once during the dry season of 2004. A detailed description of seasonal development of cyanobacteria and cyanotoxins in San Roque reservoir is given by Amé et al. (2003) and Ruibal Conti et al. (2005).

O. bonariensis (average weight: 61.11 ± 11.15 g; $n = 30$) were captured by rod fishing. Though this fish belongs to different species than those used during our laboratory study, it is ubiquitous in eutrophic lakes of Argentina (and widely distributed in South America), where is captured by both sport and professional fishermen. Thus, this fish has dietary importance in South America. On the contrary, especially adults of *O. bonariensis* are not easily maintained in aquaria.

After capture, fish were sacrificed, ice-cooled, and transported to the laboratory, where they were immediately dissected, and liver, gills, brain, intestine and muscle (ca. 5 g for each fish) separated. Gastrointestinal contents were removed and intestines washed with a physiological solution to determine the presence of cyanobacterial cells by microscopy. Five specimens were pooled to form a group. Six different groups (three from each capture) were used for MC extraction.

Bloom samples were concentrated using a nylon cloth (10 μm separation), ice-cooled and transported to the laboratory within 2 h after sampling. Afterwards, bloom material was freeze-dried and stored at −20 °C

until MC analysis. MC was extracted from water samples immediately after arrival to the laboratory following a procedure previously described (Amé et al., 2003).

In addition to fish and bloom collection, we determined the environmental conditions of sampling area by measuring water quality parameters: pH, temperature (°C), conductivity ($\mu S\,cm^{-1}$), dissolved oxygen (mg/L), suspended and dissolved solids (mg/L), ammonia (mg/L), 5-days biological oxygen demand (BOD, mg/L). Water was sampled from the surface (50 cm depth), ice-cooled, transported to the laboratory, and analyzed according to standard procedures utilizing our previous experience (Pesce and Wunderlin, 2000; Amé et al., 2003).

2.3. Microcystin analysis

Determination of cellular MC was carried out according to Amé et al. (2003). Briefly, 20 mg of freeze-dried cells were placed in Eppendorf tubes, extracted with 1.5 mL of 5% acetic acid and sonicated for 5 min in an ultrasonic bath (ULTRASONIC 300). The suspension was centrifuged at 9300 × *g* (centrifuge CAVOUR-VT1675) for 3 min, supernatant was retained and the pellet re-extracted as before. Combined supernatants were centrifuged at 9300 × *g* for 10 min. Centrifuged supernatants were applied to a C-18 solid phase extraction cartridge (LiChrolut RP-18, 500 mg, Merck), which was previously conditioned with methanol (10 mL) and 5% acetic acid (10 mL). The cartridge was washed with 10 mL of 10, 20 and 30% aqueous methanol and toxins were eluted with 3 mL of pure methanol. The eluate was evaporated to dryness under reduced pressure (40 °C, 0.3 Torr) and resuspended in 200 μL of methanol prior to high performance liquid chromatography (HPLC) analysis, carried out as described in literature (Meriluoto, 1997; Amé et al., 2003).

For determination of free dissolved MC-RR, water samples (0.5 L) were conditioned with 5% acetic acid and applied to C-18 solid phase extraction cartridge (LiChrolut RP-18, 500 mg, Merck) previously washed with methanol and further conditioned with 5% acetic acid. Toxins were eluted using methanol (3 mL, HPLC grade). The eluate was evaporated to dryness at 40 °C, suspended in 200 μL methanol (HPLC grade), and analyzed to evaluate MC-RR by HPLC (KONIK KNK500A system, UV detection at 238 nm) according to Amé et al. (2003).

For MC extraction from tissues, the method of Krienitz et al. (2003) was used with modifications. Tissues (fresh weight) were homogenized with 70% methanol containing 1% (v/v) trifluoracetic acid, using an Ultra-Turrax homogenizer. Homogenates were introduced for 5 min in an ultrasonic bath (Ultrasonic 300), followed by centrifugation at 12,096 × *g* for 10 min. Supernatants were separated and evaporated to dryness at 40 °C under reduced pressure. Dried residues of liver, gills, brain, intestine, gallbladder, and blood from *C. paleatus* and *J. multidentata* were suspended in 200 μL of methanol (HPLC-grade) prior to MC analysis. Dried residues of muscle from *C. paleatus* and *J. multidentata* as well as all tissues from *O. bonariensis* were added to 5% acetic acid (10 mL for *C. paleatus* and *J. multidentata*, and 20–50 mL for tissues of *O. bonariensis*). Acid extracts were applied to a C-18 solid phase extraction cartridge (LiChrolut RP-18, 500 mg, Merck), previously washed with methanol and further conditioned with 5% acetic acid. Microcystins were eluted with methanol (3 mL, HPLC grade). The eluate was evaporated to dryness at 40 °C and resuspended in 200 μL methanol (HPLC grade) (Amé et al., 2003).

Extracts of fish tissues were analyzed by high performance liquid chromatography (HPLC, KONIK KNK500A system, with UV detection at 238 nm) according to Amé et al. (2003), using standards prepared from pure MC-RR (purity >95% Sigma–Aldrich, USA). The concentration of MC-RR in tissues is expressed per gram of fresh weight. For the laboratory experiments, this amount is related to the concentration of the exposure medium (50 μg/L, equaling 0.05 μg/g), to calculate accumulation within 24 h exposure.

Tissue extracts were furthermore analysed by liquid chromatography–mass spectrometry (LC–MS), performed on an electrospray time-of-flight instrument (ESI-TOF, Mariner, Applied Biosystems, Darmstadt, Germany) operating in the positive mode. The high voltage was 3800 V, the nozzle voltage was 75 V, and the nozzle temperature was 140 °C. The instrument was calibrated for exact mass measurements using the triply charged ions at *m*/*z* 432.8998 and 558.3105 of the peptides angiotensin I and neurotensin, respectively. A capillary LC system (UltiMate, Dionex) was coupled online to the electrospray source. The elu-

ate of the capillary LC column was transferred to the electrospray source without splitting. Five microliters of the samples dissolved in 0.2% formic acid (FA) in acetonitrile–water (1:9, v/v) were injected. Chromatographic runs were performed using a capillary C18 column (PepMap C18, 3 μm, 15 cm × 300 μm i.d., Dionex), a linear gradient of 5–80% B in 48 min, and an eluent flow rate of 4 μL/min. Mobile phase A was 0.1% (v/v) FA in acetonitrile–water (5:95, v/v) and B was 0.1% FA in acetonitrile–water (8:2, v/v).

The evaluation of analytical parameters was performed using five concentrations of analytical standards (0.75, 2.5, 5, 7.5 and 10 μg/mL) prepared from pure MC-RR (purity >95% Sigma–Aldrich, USA), injected six times each. Calibration plot was constructed by linear regression ($y = 51.304x + 3.295$; $R^2 = 0.993$; R.S.D. = 1.8%). The limit of detection (LOD) was taken at a signal to noise ratio of 3 (S/N = 3), while the limit of quantification (LOQ) was taken as S/N = 10. This calibration procedure affords a LOD = 0.2 μg/mL and a LOQ = 0.6 μg/mL in the injected solution, which are in good agreement with values reported in the literature (Moollan et al., 1996). The LOD and LOQ, corresponding to each analyzed organ, depend on the weight of tissue used during the extraction and clean up procedure. During the analysis of *J. multidentata* and *C. paleatus* we used at least 0.1 g of liver, brain, gallbladder and blood, arising from a pool of five fish, thus giving a calculated LOD of 0.40 and a calculated LOQ of 1.20 μg/g fresh tissue. Pooled intestines and gills of these species allowed to use at least 0.25 g fresh weight (calculated LOD = 0.16 μg/g, LOQ = 0.48 μg/g), while at least 3.5 g of pooled muscles were used (calculated LOD = 0.011 μg/g, LOQ = 0.034 μg/g). On the other hand, *O. bonariensis* allowed the use of bigger amounts of tissue: 2.5 g of pooled livers and intestines (calculated LOD = 0.016 μg/g, LOQ = 0.048 μg/g), 0.4 g pooled brains (calculated LOD = 0.10 μg/g, LOQ = 0.30 μg/g); 7 g of pooled gills (calculated LOD = 0.006 μg/g, LOQ = 0.018 μg/g); 25 g of pooled muscles (calculated LOD = 0.0016 μg/g, LOQ = 0.0048 μg/g).

Recovery percentages were evaluated from spiked samples. Thus, either ultrapure or San Roque reservoir water samples were spiked with 5, 10 and 20 μg/L pure MC-RR, followed by SPE extraction and further HPLC analysis. Recovery percentage in water samples was always over 85%. On the other hand, recovery from organs was evaluated using 0.5 g muscle of *O. bonariensis* (free of MC-RR), homogenized as previously described, and spiked with pure MC-RR equivalent to 0.5 and 1 μg/g fresh weight, affording recoveries over 80%. All recovery analyses were run by duplicate.

3. Results

3.1. Laboratory study

Both high performance liquid chromatography (HPLC) and liquid chromatography-electrospray ionization-time-of-flight–mass spectrometry (LC-ESI-TOF–MS) analyses showed the presence of MC-RR in liver, gills, brain and muscle of *J. multidentata*, whereas in *C. paleatus* MC-RR was present in liver, gills, intestine and muscle of exposed fish (Table 1).

J. multidentata showed the highest concentration of MC-RR (μg/g fresh weight) in liver, followed by gills, muscle and traces in the brain (Fig. 1A). To confirm the presence of MC-RR, selected samples were also analyzed by LC-ESI-TOF–MS. Since a mass resolution of 6000 is routinely available this method enables the determination of exact monoisotopic masses of MC with accuracy above 20 ppm. In combination with

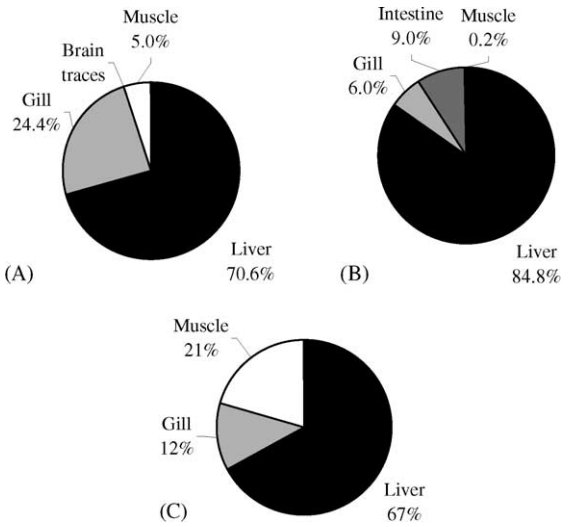

Fig. 1. Relative distribution of MC-RR in different tissues of fish: (A) *Jenynsia multidentata*, (B) *Corydoras paleatus* and (C) *Odontesthes bonariensis*.

Table 1
Presence of MC-RR in different tissues experimentally exposed to dissolved toxin (*J. multidentata* and *C. paleatus*) or naturally exposed to toxic cyanobacterial blooms (*O. bonariensis*)

Fish species	MC-RR in fish tissue (μg/g fw) (accumulation factor)						
	Liver	Gill	Brain	Muscle	Intestine	Gallbladder	Blood
J. multidentata	1.62 ± 0.73 (32.4)	0.56 ± 0.15 (11.2)	Traces (<LOQ)	0.11 ± 0.04 (2.2)	<LOD	<LOD	No blood sample
C. paleatus	19.63 ± 10.03 (392.6)	1.40 ± 0.50 (28.0)	<LOD	0.04 ± 0.01 (0.8)	2.09 ± 0.40 (41.8)	<LOD	<LOD
O. bonariensis	ds: 0.05 ± 0.05	ds: 0.02 ± 0.03	<LOD	ds: 0.01 ± 0.01	ds: cyan. cells	Not evaluated	Not evaluated
	ws: 0.38 ± 0.55	ws: 0.04 ± 0.05		ws: 0.13 ± 0.18	ws: cyan. cells		
	year: 0.16 ± 0.32	year: 0.03 ± 0.03		av: 0.05 ± 0.11			
	Min.: <LOD	Min.: <LOD		Min.: <LOD			
	Max.: 1.01	Max.: 0.10		Max.: 0.34			

Samples were analyzed by HPLC, and 18 samples additionally by LC-ESI-TOF. Reported values correspond to the mean (±S.D.) and are given in μg MC-RR/g fw of the tissue. Accumulation factor is given within parentheses. <LOD, below detection limit; <LOQ, below quantification limit; ds, dry season; ws, wet season.

capillary LC ESI-TOF, mass spectrometry was particularly useful to verify the presence of MC-RR in the brain of *J. multidentata*, where UV-HPLC detected only trace amounts. Fig. 2 shows the LC–MS analysis of brain tissue of *J. multidentata*. The extracted ion chromatogram (Fig. 2A) which discloses only the ion intensities of the doubly and singly charged ions of MC-RR revealed a distinct peak at a retention time of 28.1 min. Because of the two basic arginine residues which are preferred protonation sites, the corresponding mass spectrum (Fig. 2B) shows exclusively the doubly charged $[M + 2H]^{2+}$ ion at *m*/*z* 519.79 (calculated *m*/*z* 519.79).

C. paleatus also accumulated the highest concentration of MC-RR in liver, followed by intestine, gills and muscle (Figs. 1B and 3). Although both species were exposed for the same time to the same concentration of MC-RR, the total amounts of accumulated toxin differed markedly. The main difference between the two species were: (a) the brain, where traces were detectable in *J. multidentata* but not in *C. paleatus*, and (b) the intestine, into which *C. paleatus* absorbed more toxin than into the gills, whereas in the intestine of *J. multidentata* no toxin was evident. Furthermore, toxin accumulation to the liver of *C. paleatus* was more than tenfold compared to the liver of *J. multidentata*, and into the gill tissue more than 2.5-fold, whereas only one third was taken up into the muscles (Table 1).

3.2. Field study

Water quality and the content of cyanobacterial toxins in wet and dry season are reported in Table 2, showing a seasonality of most parameters. Water was always fully saturated with oxygen. During the wet season, turbidity was increased by suspended solids, mainly consisting of cyanobacterial bloom material. This contributed to increased pH and biological oxygen demand. Conductivity is lower during the wet than dry season, presumably because of dilution. At the end of the rainy season, growth of cyanobacteria was supported due to a combination of multiple factors such as appropriate temperature, high nutrient loads, and good irradiation. As a result of cyanobacterial mass development both cyanotoxin production per dry weight of cells and traces of cyanotoxin released in the water were evident (Table 1). On the other hand, an increase in the amount of ammonia during the dry season is related

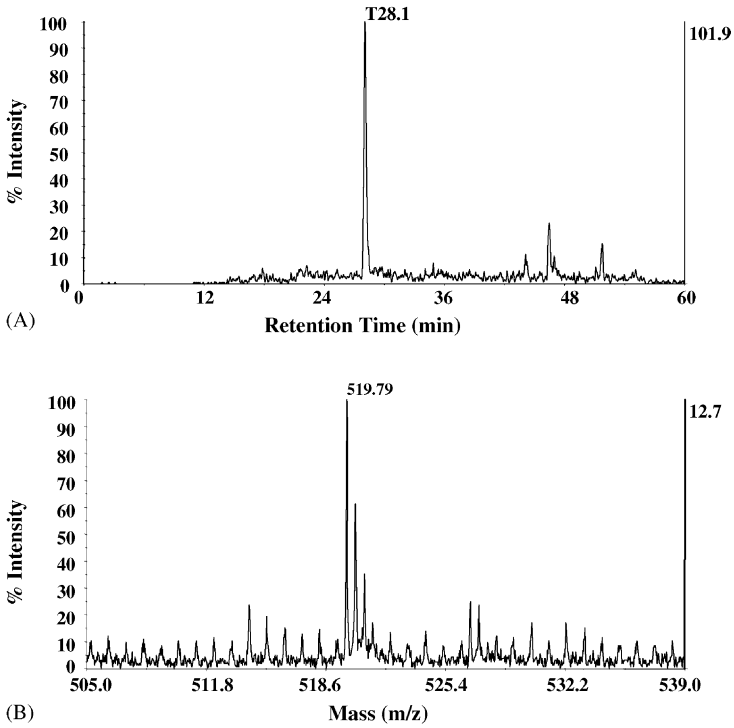

Fig. 2. LC-ESI-TOF mass spectrometry detection of microcystin-RR of brain tissue of *J. multidentata* experimentally exposed to MC-RR. (A) Extracted ion chromatogram (*m/z* 519.8, 1038.6) of microcystin-RR. (B) Mass spectrum which shows the doubly charged ion of microcystin-RR eluted at the retention time of 28.1 min.

to a drop in MC production. All of these results are in good agreement with our previous report (Amé et al., 2003). Further research is under way to elucidate if the increase in the production of MC is due to environmental factors, or if the dominance of phytoplankton communities is seasonally changing towards a dominance of toxic strains within the bloom (ecological factor).

Table 2
Water quality and MC-RR concentration in water and bloom material from San Roque reservoir, Argentina, in dry and wet season of 2004

Parameter	Season		
	Wet	Dry	Average
Temperature (°C)	14.3	12.0	13.15
pH	9.42	8.62	9.02
Dissolved oxygen (mg/L)	15.50	10.95	13.23
BOD (mg/L)	10.90	3.58	7.24
Ammonia (mg/L)	0.37	0.89	0.63
Solid suspended (mg/L)	373	43	208
Solid dissolved (mg/L)	200	206	203
Conductivity (μSi cm^{-1})	197	332	265
Dissolved MC-RR (μg/L)	<LOQ	<LOD	–
Cellular MC-RR (μg/g)	41.59	9.65	25.62

<LOD, below detection limit; <LOQ, below quantification limit.

Analyses of gastrointestinal tract of *O. bonariensis* showed presence of cyanobacterial cells, evidencing the ingestion of bloom material by this fish species. MC-RR was found in liver, gill and muscle of *O. bonariensis* (Table 1), but not in intestine tissue. The relative distribution of MC-RR in *O. bonariensis*, shown in Fig. 1C, revealed highest amounts in liver, followed by muscle and gills. Compared to muscle tissues of the other investigated fish species, *O. bonariensis* muscle accumulated highest amounts of cyanotoxins. An analysis of seasonal differences showed that the highest concentrations of MC-RR in bloom material and fish tissues occurred in the wet season (Tables 1 and 2). Particularly, the concentration of MC-RR recovered from muscle ranged from traces during he dry season to a maximum of 0.339 μg/g during the wet season.

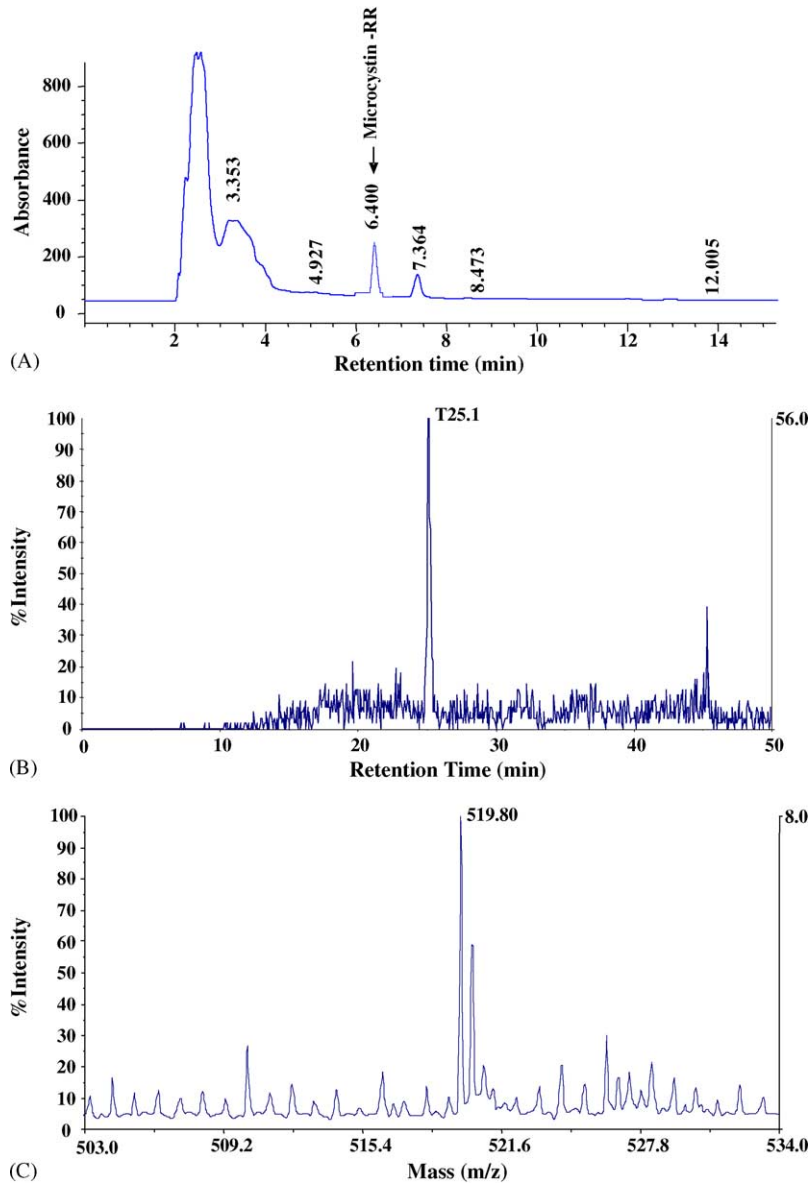

Fig. 3. Chromatograms measured in the liver of *C. paleatus*. (A) HPLC chromatogram monitored at 238 nm. (B) Extracted ion chromatogram (*m*/*z* 519.8, 1038.6) of microcystin-RR. (C) Mass spectrum which shows the doubly charged ion of microcystin-RR eluted at the retention time of 25.1 min.

4. Discussion

4.1. Uptake of MC-RR by fish

In the aquatic environment, cyanotoxins are mainly released to the surrounding water during cyanobacterial cell senescence, death and lysis. Thus, fish are naturally exposed to MC through ingestion of toxic cyanobacteria or contaminated food, and, to a less extent, through dissolved toxin. Hypothetically, two uptake routes are possible: MC-RR can be absorbed through either gastrointestinal tract or gills.

Although the fish were not fed during their laboratory exposure, we found presence of variable amounts of MC-RR in gills as well as in internal tissues of fish exposed to dissolved MC during 24 h (*J. multidentata* and *C. paleatus*; Table 1). On the one hand, the exposure solution, containing MC-RR could be drunk by *C. paleatus* and *J. multidentata* since freshwater fish can drink water, though at lower rates than marine fish do (Fuentes and Eddy, 1997; Best et al., 2003). This assumption moreover requires that MC is absorbed through the gastrointestinal tract of fish. Tencalla and Dietrich (1997) proved that the absorption of microcystin from intestine to blood system occurred rapidly in rainbow trout fed with *Microcystis aeruginosa* (concentrations peaked 3 h after forced feeding). Thus, here is good evidence that MC can be taken up through the gastrointestinal tract of fish by either drinking water or contaminated food.

On the other hand, MC-RR may be taken up via gills, during ventilation. However, the permeability of gill membranes to MC is discussed controversially. Some authors suggest that epithelia of gills and skin of freshwater fish form a barrier to microcystin transport (Andersen et al., 1993; Tencalla et al., 1994; Bury et al., 1995). Nevertheless, damage of gills by MC-LR, which has been observed during experiments with tilapia and trout (Gaete et al., 1994; Bury et al., 1996, 1998a; Zambrano and Canelo, 1996; Chorus and Bartram, 1999), suggests enhancement of microcystin uptake. Recently, Xie et al. (2005) also propose MC uptake through routes other than the gastrointestinal tract in carnivorous fish (e.g. via gills). Though further studies are required to confirm the uptake of MC through gills, our experiments evidenced the presence of this toxin in this tissue. This may show gills as a route for the entry of MC into fish body.

During our field study, we detected MC-RR in both external (gills) and internal tissues (liver, muscle) of *O. bonariensis*. As we observed cyanobacteria cells in the gastrointestinal content, the ingestion can be proposed as the most probable route of toxin uptake in this wild fish. Best et al. (2003) have found that exposure of fish to the contents of cyanobacteria (lipopolysaccharides, LPS) stimulates the drinking response, which potentially increases the opportunity for the uptake of toxins. Cyanobacterial LPS should not be present in the laboratory study, but could be responsible for enhanced drinking of the fish in the field study. However, we also recorded dissolved MC-RR in the water where fish were captured (Table 1). Thus, MC uptake via gills would not be discarded as discussed for experimental exposure of *J. multidentata* and *C. paleatus*.

4.2. Distribution of MC-RR in fish

Once absorbed by either gills or intestinal epithelia, a rapid transport of the toxin throughout the fish body may take place. Thus, MC can be transported via the bloodstream and distributed to various organs or tissues. Because blood is in contact with all tissues, the toxin is distributed especially in organs like gills, liver, intestine, kidney, etc., which are highly blood-irrigated.

During this study we did not detect MC-RR in blood of *C. paleatus* but in internal organs/tissues (liver and muscle) of this fish (Table 1). This evidences that at least part of the toxin is capable of entering the blood stream (via the intestine, gills, or by other mechanism), with subsequent distribution to various body tissues. The presence of MC in the blood of fish has been demonstrated by Tencalla and Dietrich (1997), and Xie et al. (2004, 2005).

4.3. Accumulation of MC-RR in fish

4.3.1. Liver

Microcystins mainly accumulate in the liver of vertebrates, but in several fish species (Atlantic salmon, rainbow trout, goldfish, common carp, silver carp, tilapia) under laboratory conditions utilizing different routes of exposure (intraperitoneal injection and feeding), accumulation has also been demonstrated in kidney, intestine, gallbladder, gills and muscle (Williams et al., 1995; Tencalla and Dietrich, 1997; Bury et al., 1998b; Malbrouck et al., 2003; Jang et al., 2004; Li et al., 2004; Soares et al., 2004; Xie et al., 2004). For instance, a rapid and high accumulation of MC-LR was observed in liver of Atlantic salmon and rainbow trout exposed to radiolabeled MC-LR, administrated via intraperitoneal injection or by oral gavaging, respectively (Williams et al., 1995; Tencalla and Dietrich, 1997). Moreover, field studies have also detected MC in liver of fish (Magalhaes et al., 2001, 2003; Sipiä et al., 2001; Mohamed et al., 2003; Xie et al., 2005). The liver is the most severely affected organ in fish exposed to MC (Råbergh et al., 1991; Carbis et al., 1996; Kotak et al., 1996; Fischer and Dietrich,

2000a; Fischer et al., 2000). Microcystin accumulation into the liver is mediated by a highly expressed unspecific organic anion transporter (bile acid carrier transport system) (Runnegar et al., 1991; Fischer et al., 2005). From the liver, it can be either reintroduced into the intestine by entero-hepatic recirculation (Falconer et al., 1992) or conjugated during the detoxification process (Pflugmacher et al., 1998).

We have found the highest concentration of MC-RR in liver of both experimentally exposed and wild fish (Table 1, Fig. 1), which is in good agreement with the previously discussed literature.

Moreover, during identical exposure conditions, *C. paleatus* accumulated much higher total amounts of MC-RR into gill, liver and intestine tissue, compared to *J. multidentata*. The reason for this is possibly based on less potent biotransformation capacities in *C. paleatus*, as observed in a recent work (Cazenave et al., 2005b). Lower biotransformation rates might cause lower excretion of the metabolized toxin, hence accumulation occurs at higher level. To fully elucidate these findings, further research is needed regarding comparison of the biotransformation capacities in both species.

4.3.2. Intestine

During our study, we also observed MC-RR in the intestine of *C. paleatus* but not in its gallbladder. This could be due to the water drunk by the fish as previously discussed. At this point it is important to remark that the callichthyid catfishes of the genus *Corydoras* are air-breathing, using their intestine as gas exchange organ (Kramer and McClure, 1980; Gómez, 1993). In order to air-breathe via the intestine, fish suck air, possible accompanied by some water, into the intestine via the anus. Accordingly, the intestine of *Corydoras* is highly vascularized in comparison with the corresponding tissue in non-air-breathing species, like *J. multidentata* or *O. bonariensis*, and is thus a place of high resorption capacity. This fact could explain why we found MC-RR in the intestine of *C. paleatus* but not in intestines of *J. multidentata* or *O. bonariensis* (Table 1). Furthermore, it might be one reason for the much higher total amount of absorbed toxin in *C. paleatus*, compared to the other two species.

On the other hand, the presence of microcystin in the intestine could be a result of desorption from the blood stream. This suggestion is based on the premise that influx and efflux of microcystin across membranes of perfused organs might occur. Thus, the intestine and gills, which are highly vascularized, might receive microcystin from blood for its excretion (by faeces or during ventilation). Williams et al. (1995) have found toxin in the digestive tract and in gills of Atlantic salmon after intraperitoneal dosing with tritiated dehydromicrocystin-LR, which confirms that MC is able to cross membranes from the blood system to the intestine and also to gills. Redistribution of MC-RR to the gills is the second possible mechanism besides direct uptake from the media in all studies species (Table 1). Even in gills of mussels fed with toxic *Microcystis* strain, a low percentage of MC-LR was present (Vasconcelos, 1995).

To this point, it seems evident that MC might be absorbed by the intestine, gills and probably other exposed tissues of fish, transported by blood, and accumulated in different organs.

4.3.3. Muscle

The study of the uptake and accumulation of MC by fish has received considerable attention, mainly arising from the concern for the effects of MC on humans. Hence, several laboratory and field studies have shown that MC is accumulated in muscle of different fish species (common carp, tilapia, Atlantic salmon, rainbow trout), although the concentration of MC in muscle is usually much lower than that in other tissues (Williams et al., 1995; Bury et al., 1998b; Magalhaes et al., 2001, 2003; Li et al., 2004; Soares et al., 2004; Xie et al., 2005).

Our laboratory results show that MC-RR can be present in muscle of fish after 24 h exposure, whereas the field study shows that MC-RR is also present in muscle of wild fish chronically exposed to toxic blooms (Table 1).

Fig. 1 presents the relative distribution of MC-RR to different tissues on both laboratory and field exposed fish. Despite of the predominance of MC-RR in liver, it is interesting to note the relative abundance of MC-RR in muscle of fish exposed for 24 h under laboratory conditions (Fig. 1A and B) in comparison with wild fish chronically exposed (Fig. 1C). It seems that accumulation of MC-RR in muscle begins shortly after exposure, and increases with time leading to an increased amount of MC-RR in muscle of chronically exposed fish. This is particularly dangerous for human health, mainly

because MC might be transferred through the food web from contaminated fish to consumers. Considering the average value for MC-RR in muscle of *O. bonariensis* (Table 1), and assuming a 70 kg person eating 100 g fish muscle per day, a calculation reveals an average consumption of 5 μg MC-RR per day, which exceeds the tolerable daily intake (TDI) of 0.04 μg/kg body weight per day, recommended by the World Health Organization (WHO, 1998). This situation is obviously worse for kids or people having less body weight. The risk of consumption of contaminated fish increases during wet season, where we observed the highest concentrations of MC-RR in muscle (Table 1). This last result suggests the need of a worldwide action to prevent the consumption offish contaminated with MC.

4.3.4. Brain

Recent laboratory experiments with prawn indicate that brain was one of primary organs for accumulation of nodularin (Kankaanpää et al., 2005). First evidence of MC-LR accumulation into the brain offish was given by Fischer and Dietrich (2000b), detecting the toxin by western blot 48 h after bolus dosing of freeze dried algae equivalent to 400 μg/kg of carp (*Cyprinus carpio*). We add to this knowledge by reporting the presence of MC-RR in brain of *J. multidentata*, exposed to water-dissolved toxin. The presence of MC-RR in brain of *J. multidentata* requires the crossing of the blood–brain barrier. Recently, Fischer et al. (2005) provided evidence that members of the organic anion transporting polypeptide superfamily (OATPs) are capable to transport MC-LR across the blood–brain barrier of the frog *Xenopus laevis*. This finding and our results give strong evidences on the ability of MC to cross through the blood–brain barrier and rose questions on the probable neurotoxicity of MC, especially if we consider that fish exposed to MC-LR evidenced behavioral changes (Baganz et al., 1998, 2004).

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