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BIOACCUMULATION OF MICROCYSTINS BY FISH ASSOCIATED WITH A PERSISTENT CYANOBACTERIAL BLOOM IN LAGO DE PATZCUARO (MICHOACAN, MEXICO)

JOHN P. BERRY,*† ELISHA LEE,† KATHERINE WALTON,† ALAN E. WILSON,‡ and FERNANDO BERNAL-BROOKS§
†Department of Chemistry and Biochemistry, Florida International University, North Miami, Florida, USA
‡Department of Fisheries and Allied Aquacultures, Auburn University, Auburn, Alabama, USA
§Instituto de Investigaciones sobre los Recursos Naturales, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, Mexico

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Abstract—Lago de Patzcuaro is a historically important freshwater fishery in Mexico. The lake is presently characterized by a persistent bloom of cyanobacteria, specifically dominated by recognized producers of toxic microcystins (MCYSTs). We evaluated MCYSTs in sestonic and dissolved fractions of the water column, as well as representative fish species (silversides, *Chirostoma* spp.; *Goodea* sp.; and carp, *Cyprinus carpio*) obtained from local markets and small commercial catches during the bloom. Samples were evaluated primarily by enzyme-linked immunosorbent assay (ELISA), and secondarily by protein phosphatase (PPase) inhibition assay and liquid chromatography-mass spectrometry (LC-MS). Sestonic MCYST concentration (0.02–0.36 μg/L) generally correlated inversely with distance from the bloom, supporting the bloom as the source of the toxin. Several MCYST variants, including MC-LR, -LA and -LY, as well as didemethyl variants, were identified by LC-MS/MS analysis. All three species of fish bioaccumulated MCYSTs in relevant tissues, and toxin content correlated with trophic level, with highest and lowest levels measured in phytoplanktivorous and zooplanktivorous representatives, respectively. Detection of MCYST in silversides and *Goodea* sp. is particularly relevant because both are consumed in their entirety, including viscera (e.g., liver) known to primarily accumulate MCYST. These results indicate that Lago de Patzcuaro is indeed characterized by a toxigenic bloom, and that commercially important fish species from the lake accumulate toxic MCYST in tissues relevant to human consumption. As such, this system may represent an ideal model of the trophic transfer of MCYSTs and its relevance to human and environmental health. Environ. Toxicol. Chem. 2011;30:1621–1628. © 2011 SETAC

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INTRODUCTION

Cyanobacteria (blue-green algae) produce a diversity of toxic or otherwise bioactive metabolites, including a number of toxins that have been associated with human and environmental health concerns [1]. In particular, contamination of drinking water by toxigenic cyanobacteria, as a direct route of exposure to these toxins, has been rather well documented [1]. Although much less well studied, a growing body of evidence ([2–8] and others) suggests that cyanobacterial toxins may also bioaccumulate in freshwater food webs, posing a largely uncharacterized route of human exposure to these metabolites.

Perhaps the best studied, and arguably most widespread, of the freshwater cyanobacterial toxins are the microcystins (MCYSTs). Accumulating primarily in the liver, specifically via organic anion transporters [9], the MCYSTs have been linked to human illness, and even death, as hepatotoxins [1]. Chemically, the MCYSTs—of which over 80 variants have been reported [10]—are cyclic heptapeptides with a core peptide structure, including the unique β-amino acid, (*2S, 3S, 8S,9S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic (Adda), and two variable amino acid positions that are primarily responsible for the structural diversity within the family. Toxicologically, the MCYSTs are potent inhibitors of type 1 and 2a Ser/Thr protein phosphatase, to which they bind both reversibly and covalently [11]. In particular, contamination of drinking water, and related direct routes of ingestion, have been implicated and subsequently documented in cases of human and animal intoxication by MCYSTs. However, several recent studies have suggested that MCYSTs, like other algal toxins, can accumulate in fish and other components of freshwater food webs [2–5]. That said, little is known with respect to the possible health effects, especially to humans, of MCYSTs in the food web; in particular, this has been largely limited because of the lack of a sufficient model system for studying diet-derived MCYST.

Lago de Patzcuaro is a part of an endorheic tecto-volcanic basin located in the state of Michoacan, Mexico. Historically, the lake has been an important freshwater fishery, and it is particularly renowned as a source of the regional delicacy, *pez blanco* (*Chirostoma estor*). Although *pez blanco* and other larger species are still fished from the lake, data [12] indicate that current productivity of the fishery is largely dominated by a high abundance of smaller fish species, particularly *Chirostoma* spp. (silversides) and various goodeids. In addition to overfishing, the lake is presently characterized as eutrophic. Specifically, studies have attributed eutrophication of the lake to several factors, including erosion and runoff from deforested uplands [13], water level declines (~3 m in the past 70 years) combined with wind-induced currents and stronger sediment resuspension in a shallower water column [14], and point-source contamination from local communities along the lake [15]. Regardless, eutrophication of Lago de Patzcuaro is notable when compared with the nearby Lago Zirahuen, which is generally regarded as oligotrophic, despite being part of the same Lerma-Chapala Basin [16].

* To whom correspondence may be addressed (berryj@fiu.edu).
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In particular, and most relevant to the present study, Lago de Patzcuaro is presently characterized by a dense, consistent bloom of cyanobacteria, dominated by *Aphanizomenon* and *Microcystis*, specifically in the deepest, northeastern region of the lake [15,16]. In previous decades, the bloom appeared from April to May [16,17], when algal growth potentials reach the maximum value, specifically in response to reduced seasonal lake water volume and flow during drought periods [15]. The bloom now persists year-round, particularly appearing on the water surface under calm meteorological conditions of mornings, and before wind-induced mixing in the afternoon [18].

Because both of the dominant cyanobacterial genera in the bloom are known producers of toxins, specifically including MCYSTs that are well documented as toxic metabolites of *Microcystis*, the question arises whether this bloom is indeed toxigenic. Moreover, given the continued commercial fishing in the lake, if MCYSTs do occur, does the toxin bioaccumulate in relevant fish species, and specifically those tissues of fish that are consumed by humans? With regard to the latter question, perhaps the most noteworthy aspect of the local practices of Lago de Patzcuaro is the extensive fishing of small fish species, including several species of *Chirostoma* and *Goodea*, which are widely sold and eaten in their entirety (including the viscera), particularly in either deep-fried or dried form. For MCYSTs that accumulate primarily in hepatocytes [9], the possible concern with respect to human exposure has been largely discounted, because levels of the toxins in the most typically eaten muscle tissues (e.g., fillets) of fish have been consequently reported, as expected, to be much lower than corresponding levels in liver tissues [2,3]. The accumulation of MCYSTs in these species, in conjunction with this local practice of consuming whole fish, therefore, would represent a potentially unique system for investigating the effects of bioaccumulated MCYSTs in the food web with respect to human health. As such, we report here on the detection of MCYSTs from Lago de Patzcuaro, and the apparent bioaccumulation of this toxin by fish species from the lake, specifically including those from commercial catches or that are eaten locally.

MATERIALS AND METHODS

Chemicals and reagents

All solvents, chemicals, and other reagents were purchased from Sigma-Aldrich or VWR International, unless otherwise noted.

Field sites and sample collection

Lago de Patzcuaro is an endorheic tecto-volcanic lake located in the State of Michoacan (19°31′ to 19°41′ and 101°32′ to 101°43′), as part of the Lerma-Chapala Basin, with water level fluctuations following the seasonal dynamics of drought (December–May) and rain (June–November), and a strong decline of some 6 m in water level from 1939 to present by postulated climatic influence [14]. The water body matches the category of circadiomictic, with mixing frequently occurring during the afternoon hours by action of wind-induced currents [19,20]. In terms of primary productivity, the lake is generally considered eutrophic. Particularly relevant to the present study, the deepest (~9–10 m), northeastern end of the lake is characterized by a dense bloom of cyanobacteria, specifically composed of *Aphanizomenon* and *Microcystis*, that persists as a visible scum on the water surface during morning hours until wind-induced mixing and dissipation in the afternoon.

Samples of the water column, including both sestonic and dissolved fractions, along with surface scum algal material, were collected from Lago de Patzcuaro for MCYST analysis during morning hours of July 2008 and June 2009. Integrated samples (surface to 3 m depth) were collected by using a stoppered polyvinyl chloride tube (5 cm diameter) from locations within the bloom (Site P1), and at locations (Sites P2, P3, and P4) along a southbound transect at distances increasingly further from the bloom (Table 1). In addition, bulk samples of surface bloom material were collected for analysis of MCYSTs by liquid chromatography-mass spectrometry (LC-MS; discussed later). The pH and Secchi depth (m) were measured on/from the sampling vessel at each collection site. To evaluate the possible contribution of MCYST in lake sediments, samples of benthic sediment were also collected, extracted, and evaluated for MCYSTs. Before extraction, collected sediments were crudely fractionated into heavy and light sediments particles based on passive settling (of the former), and subsequent centrifugation to collect the latter. For comparison, two sites (Z1 and Z2; Table 1) within the epilimnion of the nearby oligotrophic lake, Lago Zirahuen, specifically situated within the same watershed, were similarly sampled.

For assessment of MCYST bioaccumulation, species of locally consumed fish, including predominantly zooplanktivorous (silversides, *Chirostoma* sp.; collected July 2008), omnivorous (carp, *Cyprinus carpio*; collected June 2009), and phytoplanktivorous (*Goodea* sp.; collected July 2008) representatives, were obtained from small commercial catches of fisherman fishing within the bloom of Lago de Patzcuaro during water quality sampling. In addition, for *Chirostoma* spp. (locally known as charales) that are sold and eaten widely in the area, samples of uncooked, cooked (deep-fried in oil), and dried fish were obtained from local markets and vendors for MCYST analyses.

Sample preparation and MCYST extraction

For the analysis of sestonic MCYSTs, well-mixed integrated samples (1 L) were collected on GF/F filters (0.2-μm pore, 4.7-cm diameter; Millipore). The filtrate was collected to quantify the concentration of dissolved MCYSTs. Processing of water samples was done on site in the laboratory at the Centro Regional de Investigaciones Pesqueras in Patzcuaro. Filter-collected material and filtrate, as well as samples of bloom material, were stored frozen (−20°C) and transported on ice to the laboratories at Florida International University for extraction and analysis; samples were extracted within two months of collection and analyzed by enzyme-linked immunosorbent assay (ELISA) within four months.

Table 1. Sites in Lago de Patzcuaro and Lago Zirahuen (Michoacan, Mexico) sampled in the present study[a]

	Site	Location	Depth	Secchi (m)	pH
Lago de Patzcuaro	P1	N 19°39′21″ W 101°35′49″	~9 m	0.03	8.60
	P2	N 19°36′9″ W 101°38′21″	ND	0.30	8.98
	P3	N 19°34′21″ W 101°38′36″	~3 m	0.25	8.84
	P4	N 19°33′11″ W 101°37′43″	< 1 m	0.15	8.64
Lago Zirahuen	Z1	N 19°26′21″ W 101°44′47″	~40 m	3.5	7.09
	Z2	N 19°26′23″ W 101°44′31″	ND	4.0	7.62

[a] See *Introduction* section for site locations. ND indicates that measurement was not determined.

Filters were extracted in the laboratory at Florida International University using the method of Wilson et al. [3], sequentially in 75% MeOH (20 ml), followed by 75% MeOH with 0.05% glacial acetic acid (20 ml), with mechanical homogenization (PowerGen 125, Fisher Scientific); extracts were subsequently centrifuged (to remove debris) and pooled together. Sediment samples were identically extracted. Dissolved MCYSTs were evaluated directly from the filtrates. All extracts and water samples were stored at −20°C until analysis.

To evaluate bioaccumulation of MCYSTs in the food web, samples of preweighed tissues from fish collected from Lago de Patzcuaro, or obtained from local markets (see earlier discussion), were likewise solvent extracted. For *Goodea* sp., viscera, including liver, were removed and transferred to 50-ml tubes for separate extraction and analysis. For carp, livers were excised and transferred to 50-ml tubes, and fillets of fish muscle were cut into portions (~5–10 g each) for subsequent extraction and analysis. In the case of silversides, given their relatively small size, along with local traditional consumption in their entirety (see *Introduction*), whole fish were extracted. Samples were stored frozen (−20°C) or transported on ice until they could be extracted and analyzed at Florida International University; samples were extracted within 2 months and analyzed by ELISA within 4 months. Fish tissues were extracted sequentially, following the methods of Wilson et al. [3], with 75% MeOH, followed by 75% MeOH with 0.05% acetic acid, with mechanical homogenization, and subsequently centrifuged and pooled. Extracts were stored at −20°C until later analysis.

For LC-MS analysis of MCYSTs (see later discussion), samples of surface bloom material, stored frozen and transported to Florida International University on ice, were solvent extracted and subsequently fractionated by solid-phase extraction (SPE). Bloom material (17.6 g dry weight) was extracted by mechanical homogenization in a blender with 0.5% acetic acid (final concentration) in water (900 ml). The extract was filtered through cotton and centrifuged (1,000 *g*, 20 min). The resulting aqueous fraction was loaded onto a high-capacity C18 SPE cartridge (Grace-Vydac) preconditioned with 100% MeOH followed by Nanopure water. The unretained aqueous fraction (100% water) was collected, and subsequently the cartridge was eluted stepwise with 50, 70, and 100% MeOH in water (200 ml/elution).

Quantitative analysis of MCYSTs by ELISA

Microcystins in water (sestonic and dissolved), sediments, and fish tissues were analyzed by enzyme-linked immunosorbent assay (ELISA) specific for the Adda moiety (Abraxis Kits). Before analysis, aliquots (500 μl) of extracts (see earlier discussion) from particulates and fish tissues, along with samples of dissolved fractions, were taken to dryness in vacuo, and resuspended in phosphate-buffered saline (pH 7.4). Analyses were performed in duplicate using the manufacturer's instructions and read using a BioTek Synergy HT multiwell plate reader. As required, samples with concentrations above the upper limit of quantitation/linearity (5 ppb) were diluted and reanalyzed.

Calculation of bioaccumulation factors

Bioaccumulation factors (BAFs) were calculated as per Streit [21], based on the ratio of calculated tissue concentration (μg/kg) to calculated concentration of toxin (μg/L) in the particulate fraction, assuming that 1 L water equals 1 kg. Accordingly, a BAF of 1 or more indicates bioaccumulation of the toxin above levels in the water column. For BAF calculations, the highest concentration of particulate toxin (measured from within the bloom) in the lake was used to give the most conservative estimate of this value.

Evaluation of protein phosphatase inhibition by MCYSTs

Inhibition of Ser/Thr protein phosphatase (PPase) by MCYSTs in particulate and fish extracts was evaluated by a fluorometric method adapted from that of Bouaïcha et al. [22]. Samples (500 μl) of extracts were taken to dryness in vacuo, and resuspended in Nanopure water. Assays were otherwise conducted as per the published procedure [22]. Fluorescence of the product 4-methylumbelliferone was measured after 60 min incubation, using a Biotek Synergy HT microplate reader (EX/EM 360-460 nm). Assays were performed in duplicate. Percent inhibition was calculated relative to the negative (water only) controls. Above approximately 1 μg/L, PPase was 100% inhibited. The reported [22] detection limit of the assay is 0.1 μg/L.

LC-MS analysis of MCYSTs

Liquid chromatography-mass spectrometry was used to identify MCYST variants present in (extracted from) bloom material. Specifically, aliquots of SPE fractions (100% water and 50, 70, and 100% MeOH eluates) were evaluated using a Finnigan Surveyor system equipped with an LCQ Deca XP MAX electrospray ionization-ion trap mass spectrometer. Separation was achieved using a Phenomenex Luna C18 (2) column (5 μm particle size, 250 mm × 4.6 mm), employing one of two solvent systems. For 100% water, 50% MeOH, and 70% MeOH SPE fractions, separation as achieved with a gradient of 20 to 100% acetonitrile in water plus 0.01% formic acid (0–10 min from 20–50%, 10–15 min to 75%, 15–20 min to 100%, and 20–23 min at 100%). For the 100% MeOH SPE fraction, separation was achieved with a 50 to 100% gradient of acetonitrile in water plus 0.01% formic acid (0–10 min from 50 to 75%, 10–20 min to 100%, and 20–23 min at 100%). Common MCYST variants were identified based on their expected molecular ions as per previous studies [23,24] and a fragment ion (*m/z* 135.1) characteristic of the Adda found in most MCYST variants.

RESULTS

MCYSTs in Lago de Patzcuaro

Microcystins were detected by ELISA in all samples, including both sestonic and dissolved fractions, from Lago de Patzcuaro (Table 2). Given recognized variability among MCYST variants—specifically including modifications of the Adda moiety—the Adda-based ELISA, as used here, may not detect all variants of MCYST present. However, the rather typical presence of the unmodified Adda, particularly among the most common variants, provides a generally effective means to estimate total MCYSTs, and has indeed been employed in numerous past studies ([25–27]). Not surprisingly, the highest concentration (0.36 μg/L) was found in sestonic samples collected from a site (P1; see Table 1) within the bloom. Concentration of the toxin in sestonic fractions decreased relative to increasing distance from the bloom (P2 and P3), but increased slightly again at P4, a site at the shallow, southern end, furthest from the bloom. Samples collected from the shallow P4 site were characterized as per Secchi disk measurements (Table 1) by considerable amounts of resuspended sediments and the fact (see *Discussion* section) that relatively higher sestonic concentrations of MCYST measured at this site may derive from adsorbed toxin, and/or algal cells, in these sediments. In support

Table 2. Concentrations of sestonic and dissolved microcystins (MCYSTs), measured by enzyme-linked immunosorbent assay (ELISA), in Lago de Patzcuaro (Michoacan, Mexico)

	Site[a]	Particulate (μg/L)[b]	Dissolved (μg/L)[b]	PPase inhibition (% of control ± SD)[c]
Lago de Patzcuaro	P1	0.36 ± 0.01	0.16 ± 0.03	32.4% (±12.5%)
	P2	0.07 ± 0.04	0.19 ± 0.00	5.9% (±8.3%)
	P3	0.02 ± 0.01	0.19 ± 0.02	ND
	P4	0.11 ± 0.04	0.16 ± 0.02	2.9% (± 4.2%)
Lago Zirahuen	P1	ND	ND	NA
	P2	ND	ND	NA

ND indicates no MCYSTs detected within limits of detection for the ELISA (>0.15 ppb), or no observed inhibition in protein phosphatase (PPase) assay; NA indicates sample was not analyzed.
[a] P1 represents collections from within the Lago de Patzcuaro bloom (see *Introduction* section); see Table 1 for description of sites.
[b] Samples were evaluated for MCYSTs by ELISA, and concentration calculated based on Microcystin-LR (MC-LR) standard; given are average ($n = 2$) concentrations ± standard deviation (SD).
[c] Data shown for extracts of seston only.

of this, MCYSTs were detected in solvent extracts of both heavy (0.775 ± 0.460 ng/g) and light (19.0 ± 2.9 ng/g) sediment particles. Concentration of dissolved toxin (0.16–0.19 μg/L) in the lake, conversely, was relatively similar among all sites (Table 2). No MCYSTs were detected within the limits of quantitation in either sestonic or dissolved samples from Lago Zirahuen (Table 2).

Protein phosphatase inhibition of extracts from particulate material (Table 2) generally correlated ($R^2 = 0.958$) with concentration of MCYST measured by ELISA. Specifically, the highest inhibition (in which 100% inhibition is observed above approximately 1 μg/L) was observed for samples from P1 (within the bloom), and no inhibition was detected for extracts of seston from P3 (characterized by the lowest MCYST concentration).

Analysis of extracts from bloom material by LC-MS/MS identified the apparent presence of at least five MCYST variants (Table 3). Specifically, Microcystin-LA (MC-LA), -LY, and -LR, as well as didemethyl MC-LR and -RR, were detected based on molecular ions of the variants, along with presence of fragment ions (135.1) typical of the most common MCYSTs.

MCYSTs in fish from Lago de Patzcuaro

Microcystins also were detected by ELISA in all fish species evaluated (Table 4). By species, the lowest levels (18.5 ng/g) of MCYST were detected in zooplanktivorous silversides (*Chirostoma* spp.). Moreover, MCYST concentrations were only detectable, within limits of quantitation greater than 0.15 ppb of the ELISA, for one of the two samples of lake-caught *Chirostoma*. No MCYST was detected within the limits of quantitation for any of the samples of fresh, cooked, or dried *charales* obtained from local markets. The highest levels of MCYST were measured for both muscle (~157 ng/g) and viscera (~867 ng/g) from phytoplanktivorous *Goodea* sp. caught in the vicinity of the bloom. Intermediate, but appreciable, levels of MCYST were also detected in samples of both muscle (4.99 ng/g) and liver (93.6 ng/g) from omnivorous carp, likewise caught in the vicinity of the bloom. For all species, calculated BAFs indicate that MCYST is bioaccumulated at levels above those present in the water column (i.e., BAF > 1).

In addition to ELISA, MCYSTs were also indicated by PPase inhibition. In agreement with ELISA, high inhibitory activity in the PPase assay was observed for extracts from both muscle (61.8%) and liver (100%) of *Goodea* sp. No inhibition of PPase activity was measured for extracts of *Chirostoma* spp. caught from the vicinity of the bloom. Inhibitory activity was measured for samples of both cooked (10.3%) and uncooked (27.9%) *charales* obtained from the local market, although MCYST was not detected (within the limits of quantitation of the ELISA) for these samples. Samples of carp were not analyzed by PPase assay.

Table 3. Microcystin (MCYST) variants, identified by liquid chromatography-mass spectrometry (LC-MS), in the Lago de Patzcuaro (Michoacan, Mexico) cyanobacterial bloom[a]

C18 SPE Fraction	MCYST Variant	Observed ions (m/z)[b]
100% Water	MC-LA	910.5 $[M+H]^+$ and 135.1
	MC-LY	1002.5 $[M+H]^+$ and 135.1
50% MeOH	Didemethyl MC-LR	967.5 $[M+H]^+$ and 135.1
70% MeOH	Didemethyl MC-RR	505.7 $[M+2H]^{2+}$ and 135.1
100% MeOH	MC-LR	986.5 $[M+H]^+$ and 135.1

[a] Microcystin variants (i.e., MC-LA, -LY, -LR and –RR) abbreviated as per the standard naming system; the two-letter suffix indicates the amino acid present in the variable region. The protonated ($[M+H]^+$) molecular ion, the doubly protonated ($[M+2H]^+$) molecular ion, and the product ions are given here to identify variants.
[b] [23,24].

DISCUSSION

MCYSTs in Lago de Patzcuaro and associated bloom

The results of the present study confirm that cyanobacteria from the lake's persistent bloom produce appreciable, albeit relatively low, levels of MCYSTs. Notably, in comparison with the eutrophic Lago de Patzcuaro, no MCYSTs were detected in the nearby oligotrophic lake, Lago Zirahuen, which is part of the same Lerma-Chapala Basin watershed. Whereas concentrations of dissolved toxin were fairly constant throughout the epilimnion (<3 m) of the lake (Table 2), sestonic concentrations of MCYSTs are highest at sites within, and generally decrease at sites further from, the bloom (Tables 1 and 2), supporting the bloom as the source of the toxin in these samples.

An exception to this trend is the somewhat higher levels of MCYSTs in seston collected from a shallow site (P4) in the southern end of the lake. Secchi depth measurements (Table 1) decreased at this shallower site, because of higher levels of resuspended sediment, and seston from this shallow site clearly contained resuspended benthic sediments. Analysis of sediment extracts collected at this shallow site indicated that the presence of MCYSTs likely derived from either adsorbed toxin or algal cells present in the sediments. Specifically, MCYSTs were detected in sediment extracts, at concentrations well above those measured for filter-collected seston, for both light (19.0 ± 2.9 ng/g) and heavy (0.775 ± 0.460 ng/g) particles of sediments, suggesting that incorporation of these particles (and particularly the latter) into collected seston samples could

Table 4. Microcystin (MCYST) content of fish from commercial catches in Lago de Patzcuaro (Michoacan, Mexico)

Species	Tissue[a]	Source	MCYST (ng/g)[b]	Trophic level[c]	BAF[d]	PPase Inhibition (% control ± SD)
Goodea sp.	V/L	Commercial catch from bloom	867 ± 315	PP	2409	100% (± 16.6%)
	M	Commercial catch from bloom	157 ± 61.5	PP	436	61.8% (4.2%)
Carp (*Cyprinus carpio*)	L	Commercial catch from bloom	93.6 ± 45.1	OM	260	NA
	M	Commercial catch from bloom	4.99 ± 2.14	OM	14	NA
Silversides or *Charales* (*Chirostoma* sp.)	Wh	Commercial catch from bloom	18.5 ± 1.92[e]	ZP	51	ND
	Wh	Market (uncooked)	ND	ZP	NA	27.9% (± 5.6%)
	Wh	Market (cooked)	ND	ZP	NA	10.3% (± 12.1%)
	Wh	Market (dried)	ND	ZP	NA	ND

ND indicates no MCYSTs detected within limits of detection for the enzyme-linked immunosorbent assay (ELISA) (>0.15 ppb), or no observed inhibition in protein phosphatase (PPase) assay.

[a] Liver (L) and/or viscera (V) were separated from dorsal muscle (M) from *C. carpio* and *Goodea* sp. for extraction and subsequent evaluation; for *Chirostoma* spp., whole (Wh), individual fish were extracted and subsequently evaluated.

[b] Samples were evaluated for MCYSTs by ELISA.

[c] ZP = predominantly zooplanktivore; OM = omnivore; PP = phytoplanktivore.

[d] BAFs > 1 suggests MCYSTs are bioaccumulated at levels above those in the water column; NA indicates that BAF was not analyzed because of lack of information regarding levels of the toxin in the water column at collection locations.

[e] MCYST only detected (with limits of ELISA, >0.15 ppb) in one of two fish.

explain the elevated sestonic levels of MCYST at this site. Previous studies [28,29] have, in fact, shown that clay and other inorganic substrates can adsorb MCYSTs. Alternatively [30], *Microcystis* cells may overwinter or otherwise deposit themselves on the surface of sediments. Either adsorbed toxins or algal cells deposited in the resuspended sediments could explain the higher levels of particulate MCYSTs at this site, suggesting that sediments could represent a considerable source of MCYSTs in the lake.

As perhaps the most widespread of the cyanobacterial toxins, MCYSTs have been measured in freshwater systems worldwide. Only recently, however, have MCYSTs been identified in Mexican freshwater systems. In addition to a previous report in the scientific literature of MC-LR [31] in a reservoir in Valle de Bravo, two very recent studies [32,33] have identified MCYSTs or MCYST-producing strains of *Microcystis* in several such systems, including natural and urban lakes, additional reservoirs, and channels. Concentrations of particulate MCYST in Lago de Patzcuaro are low to intermediate to levels found in other freshwater systems, and lower than levels measured in other Mexican lakes. Evaluation of MCYSTs in several German lakes by Fastner et al. [34], for example, measured particulate concentrations of the toxin as high as 25 mg/L. More typically, however, particulate concentrations generally range from a few micrograms to a few nanograms per liter [25,26,34–36]. Likewise, concentrations of dissolved MCYST in Lago de Patzcuaro are typical of those measured elsewhere. For both dissolved and particulate fractions, MCYST levels are below the World Health Organization Guidelines' limit of 1 μg/L (for drinking water).

Inhibition of PPase activity correlated with measured concentrations of MCYST. The PPase assay has been used previously by other investigators [37] as a measure of MCYSTs. However, the method is generally limited in accuracy, compared with ELISA, by the possible presence of non-MCYST PPase inhibitors, and differential activity of the more than 80 MCYST variants, such that relative abundance of more or less active variants can lead to over- or underestimations, respectively, of the total MCYST concentration [37]. We used the PPase inhibition assay simply to show that bioactive forms of the toxin are indeed present, as well as to supplement the assessment of relative concentrations at the different sampling sites.

To assess which variants of MCYST were present, we collected and extracted bloom material and evaluated MCYSTs by LC-MS. Based on characteristic ions of the typically most abundant variants, we identified at least five variants in these extracts/fractions. In addition to known fragmentations associated with common variants, the *m/z* 135.1 (of the Adda moiety) was employed. As for Adda-specific ELISA, modifications of the Adda moiety may lead to certain variants being missed in this approach. However, given the relatively widespread occurrence of unmodified Adda, it provides a means to effectively screen for typical variants. Specifically, the commonly found variants MC-LR, -LA, and -LY were detected, along with the apparent presence of the didemethyl forms of MC-LR and -RR. In addition to these, extracted chromatograms (not shown) based on fragment ions found in common MCYST variants (*m/z* 135.1 from Adda) suggested that additional variants also may be present, and future investigations will pursue the purification and characterization of these.

MCYSTs in commercial catches of fish

Given the extensive commercial and subsistence fishing in Lago de Patzcuaro, including in the vicinity of the algal bloom, we evaluated bioaccumulation of the toxin by several, relevant fish species and their tissues. In part because of over-exploitation of other larger fish species (e.g., *pez blanco* [*Menidia estor estor*]) traditionally fished from the lake, the Lago de Patzcuaro fishery is particularly characterized by a number of smaller species, including representatives of the genus *Chirostoma*, and goodeids [12]. Locally important, both commercially and as a regional food item, these small fish are typically eaten in their entirety, including the viscera, which would be expected to contain the highest levels of MCYSTs, which accumulate primarily in the liver. As such, bioaccumulation of MCYST in these species would potentially represent a unique route of potential exposure to the toxin, particularly because the exposure to MCYST in fish has been generally discounted because of the primary accumulation of the toxin in the liver and associated organs (usually not eaten), and relatively lower accumulation in muscle tissues.

Indeed, we identified measurable levels of MCYST in both *Chirostoma* spp. and *Goodea* sp. samples obtained from commercial catches in the vicinity of the lake's bloom, and in both cases, BAFs indicated that the toxin was bioaccumulated at

levels above those found in the water column (Table 4). For the more commonly eaten *Chirostoma* spp., however, MCYSTs were only measured within the limits of quantitation of the ELISA for one of two samples and were found to be relatively low in this case, compared with other species. Likewise, ELISA did not detect the toxin in any of the market samples. In contrast, however, MCYST was detected at quite high levels in *Goodea*, including both muscle (157 ng/g) and viscera (867 ng/g), with higher levels of the toxin measured, as expected, in the latter tissues. Relative to other fish samples in the current study, these levels were nearly 10-fold higher than the next highest concentration in liver tissues (of *C. carpio*), or more nearly 30-fold higher than levels observed in muscle (from *C. carpio*). That said, for comparison, studies in highly eutrophic systems (e.g., Lake Chaohu, China; Septiba Bay, Brazil) have measured levels in the range of several micrograms per gram of tissue (4–5).

Microcystins were also detected in both muscle and liver of carp caught in the vicinity of the lake's bloom, and relatively much higher concentrations were, likewise, detected in the latter (Table 4). Carp from the lake is, in fact, eaten locally; however, unlike *Chirostoma* and *Goodea* species, the larger carp are not eaten in their entirety, but rather typically prepared by filleting, such that exposure to MCYST from only muscle would, in this case, be expected.

Notably, the relative levels of the toxin among the species seemed to be directly related to trophic level. Specifically, *Chirostoma* species are generally zooplanktivorous, whereas *Goodea* is a largely phytoplanktivorous genus in the lake. This suggests that accumulation of the toxin may be primarily linked to direct consumption of algal cells rather than biomagnification, or related transfer, to higher trophic levels. This is perhaps not surprising, given the water-soluble nature of MCYSTs and consequently expected lack of accumulation in fat tissues, as observed for lipophilic toxins. Although phytoplanktivorous zooplankton could provide a means of MCYST uptake by fish, the microfauna of the Lake is generally characterized (unpublished data) by small species of *Cladocera, Bosmina* and calanoid copepods, as well as rotifers that would not be expected to be particularly efficient grazers on cyanobacteria. This correlation between bioaccumulation and trophic level is further supported by the intermediate concentrations of MCYST measured in the generalist omnivore species, *C. carpio* (Table 4). Furthermore, in addition to a generally omnivorous diet, carp are recognized benthic grazers, and given the apparent presence of MCYSTs or algal cells in the benthic sediment of the Lake (as discussed previously), likely either adsorbed toxin, deposited algal cells, or invertebrate prey (as vectors of the toxin) in the benthos may contribute to the MCYST levels in this species.

In addition to ELISA, samples of *Chirostoma* spp. and *Goodea* sp. were further evaluated by a PPase inhibition assay (Table 3). Inhibition of PPase by extracts generally correlated with levels of MCYST measured by ELISA. Whereas no inhibition was observed for extracts of *Chirostoma* spp. (from the bloom), extracts from both muscle and liver of *Goodea* significantly inhibited PPase activity (Table 4) in these assays, and inhibition specifically correlated with tissue distribution of MCYST, with higher activity measured for extracts of liver than for muscle tissue. Interestingly, extracts of both cooked and raw *Chirostoma* spp., obtained from local markets, did show inhibition in the PPase assay, although ELISA did not detect MCYST within the limits of the assay. As discussed, previous studies have shown differential PPase inhibition activity for different MCYST variants, and as such, the assay was not used here to estimate MCYST content; rather, it demonstrated that bioactive variants are, in fact, present in these tissues. In the case of fish obtained from the market, possibly observed PPase inhibition may indicate, despite the lack of MCYST detection by ELISA, the presence of relatively more active variants present at levels below those detectable by the immunoassay, or alternatively, non-Adda containing variants not detected by ELISA.

With regard to levels detected by PPase assay in cooked *charales*, although MCYSTs are heat stable [38], few studies have investigated the effects of cooking on MCYST. However, one very recent study examined the effects of boiling on the amounts of extractable MCYST from intraperitoneally injected carp [39]. This study showed that boiling, in fact, significantly increased the amount of the toxin extracted from these tissues, compared with nonboiled controls from the same specimen. This was speculated to be attributable to release of MCYST covalently bound to PPases in tissues (see later discussion). Moreover, these studies [39] indicate that differential amounts of variants (comparing MC-LR with MC-RR) may be removed into the water during boiling. Specifically, in this study, relatively more of the more toxic MC-LR is retained by the tissue compared with MC-RR. Whether a similar effect might be observed by frying, as per preparation of *charales*, however, is not known.

In the current study, we did not investigate the variants present in fish tissues, particularly because of the recognized difficulties (e.g., signal suppression, toxin–biomolecule conjugates) associated with LC-MS analysis of MCYSTs in animal tissue matrices [27,40]. However, further delineation of which variants are bioaccumulated will be critical to understanding this process. In fact, a recent study by El Ghazali et al. [41] suggested, specifically using the *C. carpio* model, that relative composition of MCYST variants does indeed affect both toxicity and trophic transfer. As such, we are currently developing appropriate LC-MS methods (e.g., multiple reaction monitoring using a triple quadrupole instrument) for these future studies.

A number of previous studies have identified apparent bioaccumulation of MCYST by fish and other aquatic animal species [2–5]. Magalhães et al. [5], for example, measured MCYST levels in fish from Brazilian freshwater and marine systems. In this study, the World Health Organization Guidelines were employed to identify those species for which levels of MCYST in muscle tissues exceeded the established tolerable daily intake (TDI) limit of 0.04 μg MCYST/kg body weight. Using this approach, and similar assumptions (~300 g fish per daily serving, typical body mass of 70 kg), measured levels of MCYST in both whole *Chirostoma* spp. (~ 0.8 μg/kg) and *Goodea* spp. (>3.7 μg/kg) would exceed this tolerable daily intake. However, levels of MCYST in carp muscle tissue would fall well below (~0.02 μg/kg).

A growing body of evidence [11,27,42] suggests that most widely employed quantitative analytical methods (e.g., ELISA, LC-MS), specifically based on prior solvent extraction of the analyte, may largely underestimate total MCYST content because of recognized covalent binding between the toxin and active sites of protein phosphatase and consequent inability to solvent extract enzyme-bound toxin. Several methods for quantitative analysis of total (bound and unbound) MCYST have been developed, and studies using these techniques [27,42] indicate that conventional methods, such as ELISA and LC-MS, may underestimate total MCYST by orders of magnitude. In fact, preliminary analysis of *Goodea* samples by one such method measured approximately 10-fold higher levels of MCYST in both liver and muscle (P. Suchy and J.P. Berry,

Florida International University, unpublished data). The contribution of covalently bound MCYST as toxic contaminant has not yet been firmly established [2]; however, a very recent study [43] has shown that predicted MCYST-peptide fragments, resulting from enzymatic digestion of covalently bound toxin, are active in a PPase inhibition assay.

CONCLUSIONS

Although bioaccumulation of MCYST has been reported in several, previous studies [5–8], much remains to be clarified with respect to the potential health effects posed by this contamination. In particular, no known cases exist of acute intoxication associated with consumption of food-borne MCYSTs, although studies such as this do suggest that bioactive (PPase inhibitory) forms of the toxin are present in, and extractable from, tissues. Fisheries in which regional fishing practices (such as reliance on small planktivorous fish and fishing of eutrophic waters), as well as related consumption patterns associated with catches (e.g., consumption of these fish in their entirety), suggest a particularly high potential for exposure to food-derived cyanobacterial toxin and likely represent critical models for understanding possible health effects of the trophic toxin transfer. Accordingly, the identification of MCYST in Lago de Patzcuaro represents a potentially unique system for future study of the human and environmental health effects of cyanobacterial toxins in freshwater food webs.

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