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Original Article

Detection of microcystins in nile tilapia (*Oreochromis niloticus*) from a eutrophic pond containing *Microcystis aeruginosa* bloom in Dhaka, Bangladesh

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ABSTRACT: Cyanobacteria blooms are a very common phenomenon in eutrophic aquaculture ponds in Bangladesh. Microcystins (MCs) accumulation in different organs of the freshwater fish *Oreochromis niloticus*, collected from a eutrophic pond in Dhaka city containing heavy blooms of *Microcystis aeruginosa* were investigated using High Performance Liquid Chromatography (HPLC/MS). Among the organs tested MCs were only detected from the liver. No MCs was detected from the gut and muscle of the fish. Two variants of MCs were detected from the liver; MC-RR and MC-LF and their concentrations were 0.25µg/g and 0.22 µg/g, respectively. The total concentration of MCs (0.47 µg/g) found in *O. niloticus* was higher than the WHO tolerable daily intake (0.04 µg/kg). The present study suggested that the fish farms should be monitored for the presence of toxic cyanobacterial blooms to minimize the exposure of potent hepatotoxins to fish and humans through the food chain.

Keywords: *Microcystis; microcystins; blooms, Oreochromis niloticus; accumulation.*

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INTRODUCTION

Bloom formation due to excessive growth of certain cyanobacteria followed by the production of toxic compounds have been reported in many eutrophics to hypertrophic lakes, ponds, and rivers throughout the world[1]. Anthropogenic eutrophication and global climate change have created harmful algal blooms and contaminated surface waters all over the world. Cyanobacterial blooms and the accumulation of several toxins, called cyanotoxins, in water bodies pose ever ecological consequences with high risk to aquatic organisms and global public health[2]. The cyanotoxins are responsible for intermittent but repeated widespread poisonings of wild life, domestic animals, fish and human[3,4]. One of the most studied groups of cyanotoxins are the cyclic heptapeptides called Microcystins (MCs). There are 80 different variants of these MCs. MC-LR (Microcystin Leucine Arginine, MW 995.17) and MC-LA (Microcystin Leucine Alanine, MW 910.06) are the most common and the most toxic forms which occur more often in cyanobacterial blooms[5]. MCs have strong affinity to serine/threonine protein phosphatases thereby acting as an inhibitor of type 1 and type 2A phosphatases[6]. This cyclic peptide interacts with the mitochondria of animal cells triggering oxidative stress and apoptosis[6]. In China, incidences of primary liver cancer[7] and colorectal cancer[8] have been associated with MCs contaminated drinking water. Additionally, tumor promotion and liver injury caused by oral consumption of MCs pose serious health risks[9]. The World Health

Organization (WHO) has set a tolerable daily intake (TDI) for chronic exposure to MC-LR of 0.04 µg/kg body weight and a provisional guideline value of 1.0 µg/L MC-LR for drinking water[10]. Moreover, MCs are heat stable compounds, and neither boiling of water nor cooking fish prior to consumption is expected to reduce the potential for exposure[11]. de Magalhaes *et al.*, 2003 showed that aquatic animals can bioaccumulate MCs (cyanobacteria hepatotoxins)[12]. High concentrations of MCs have been detected in piscivorous and phytoplanktivorous fish[13]. Studies have demonstrated that MCs accumulate in several fish tissues, such as gut, liver, gills, kidney, bile, muscle, blood, heart, and brain[13,14,15,16,17].

About 307 different species of cyanobacteria have been reported from all kinds of water sources (river, canal, ponds, ditches, lakes etc.) in Bangladesh[18]. Among them 13 species frequently form blooms. MCs have been reported in freshwater ponds from different locations of the country[19] and their concentrations were well above the provisional WHO guideline value (1µg/l MC-LR). Ahmed *et al.*, 2000 first characterized MCs from a pond in Chandpur and later reported MCs from other parts of the country[19,20]. Recently Affan *et al.*, (2015) have studied 23 water sources in Mymensingh district and 22 cyanobacterial bloom samples were found while microcystin concentrations ranged from 25-82300 pg/ml[21]. Even in tap water, microcystins were detected in concentrations ranging from 30 to 32pg/ ml, which were very alarming for public health safety.

Freshwater fish farming plays an important role in rural livelihood and contributes 55% to total fish production in Bangladesh[22]. Excessive use of artificial feed and lack of scientific management create eutrophication leading to cyanobacterial bloom formation[19]. Mainly planktivorous fish (e.g., common carp) and omnivorous fish (e.g., tilapia) are popular for aquaculture. Zurawell *et al.*, 2005 stated that cyanobacteria are an important component of tropical cichilids (e.g., tilapia, *Oreochromis niloticus*) and cyprinids (e.g., silver carp, *Hypophthalmicthys molitrix*)[23]. Thus, potential health hazard from consumption of fish cultured in eutrophicated pond is a major concern. Although MCs have been detected from different aquaculture ponds and lakes in Bangladesh, the accumulation of toxins in fish tissue and their toxic effect on aquatic lives have not been documented. This paper reports the accumulation of MCs in fish tissue and evaluates the possible public health risk in the country.

MATERIALS AND METHODS

Location of pond

Sikkatuli pond (Nazira Bazar) is located at the old Dhaka city (23°43'07.2"N 90°24'24.0"E). The pond is 0.25 ha in size and is used for fish culture and some domestic purposes. *Microcystis aeruginosa* bloom is very common in this pond. Local people culture and consume fish from this pond.

Collection of *Microcystis aeruginosa* bloom and sample fish

M. aeruginosa bloom started in February 2014 and the highest cell density (95%) was observed in June 2014. The bloom sample was collected with a plankton net (no. 55 bolting silk plankton net). The concentrated samples were filtered through an 0.45µm glass fiber filter (Whatman GF/C, 47 mm diameter) and dried in an oven at 60-70˚C. The fish samples were harvested by local people. For the analyses of fish tissues five individuals of *O. niloticus* were dissected and livers, stomachs and muscles were pooled out and dried in an oven at 60-80˚C. Dried tissues were grinded in a kitchen grinder. In the original bloom sample, the cell density of *M. aeruginosa* was 3×10^4 colony/l recorded by a Sedgewick-Rafter counting chamber (S-R cell) under a compound microscope at ×400 magnifications. Water quality parameters were determined by ecological HACH test kit (Model FF2).

Extraction of toxins

Fish tissue sample

5 ml extraction solution (0.3% acetic acid in methanol/water (8:2 v/v)) was added to the dried and ground tissue samples. After sonication in an ultrasonic bath for 10 min the solution was shaken overnight. Solids were removed by centrifugation. 2.5 ml extract were concentrated to 1 ml using a rotary evaporator (180 mbar, 45°C). The concentrated extracts were frozen at -20°C for at least 3 hours. After thawing and filtering through a 0.45 µm PTFE filter the extract was ready for LC-MS/MS analysis.

Bloom filter

The GF/C filters were extracted with 2.0 ml water/methanol (50:50; v:v) per filter by ice-cooled sonication for 4 min with an ultrasonic probe GM 70 (Bandelin, Berlin, Germany). The extract was sonicated for another 15 min in an ultrasonic bath. Extracts were centrifuged (10000 g, 15min) and the supernatants were filtered using 0.22 µm nylon syringe filters (Roth, Karlsruhe, Germany). The extracts were directly subjected to the liquid chromatography.

Chemical analysis

The HPLC/UV determination of microcystins was carried out following the method of Lawton *et al.* (1994) with some modifications[24]. Separation was performed using a C18 column (Phenomenex prodigy, ODS (3), 250 x 4.6mm, 5 µm) and acetonitrile /water/0.05% TFA as the mobile phase. Microcystins were detected using an UV detector (Shimadzu SPD-10AV; λ=238 nm). HPLC/MS-MS analyses were applied to confirm the identity of the toxins. HPLC-MS/MS was performed using Shimadzu HPLC LC-20A coupled to an AB Sciex 4000 Qtrap with an electrospray interface. Analytes were separated on a Phenomenex Gemini 5 µm C18 column (150*3 mm) with a guard column and a mobile phase consisting of

5 mM ammonium acetate in water/acetic acid (99:1; v/v) (eluent A) and 5 mM ammonium acetate in methanol/water/acetic acid (97:2:1; v/v/v) with a flow rate of 0.4 ml/min. Elution started with 60% eluent A and 40% eluent B.

Quantification

Since reference materials for desmethylated MCs are not available commercially, determination of the concentrations of desmethyl-MCs, [D-Asp3, Dha7] MC-LR, and [Dha7] MC-LR, was performed using the standard calibration curves of MC-LR.

Chemicals

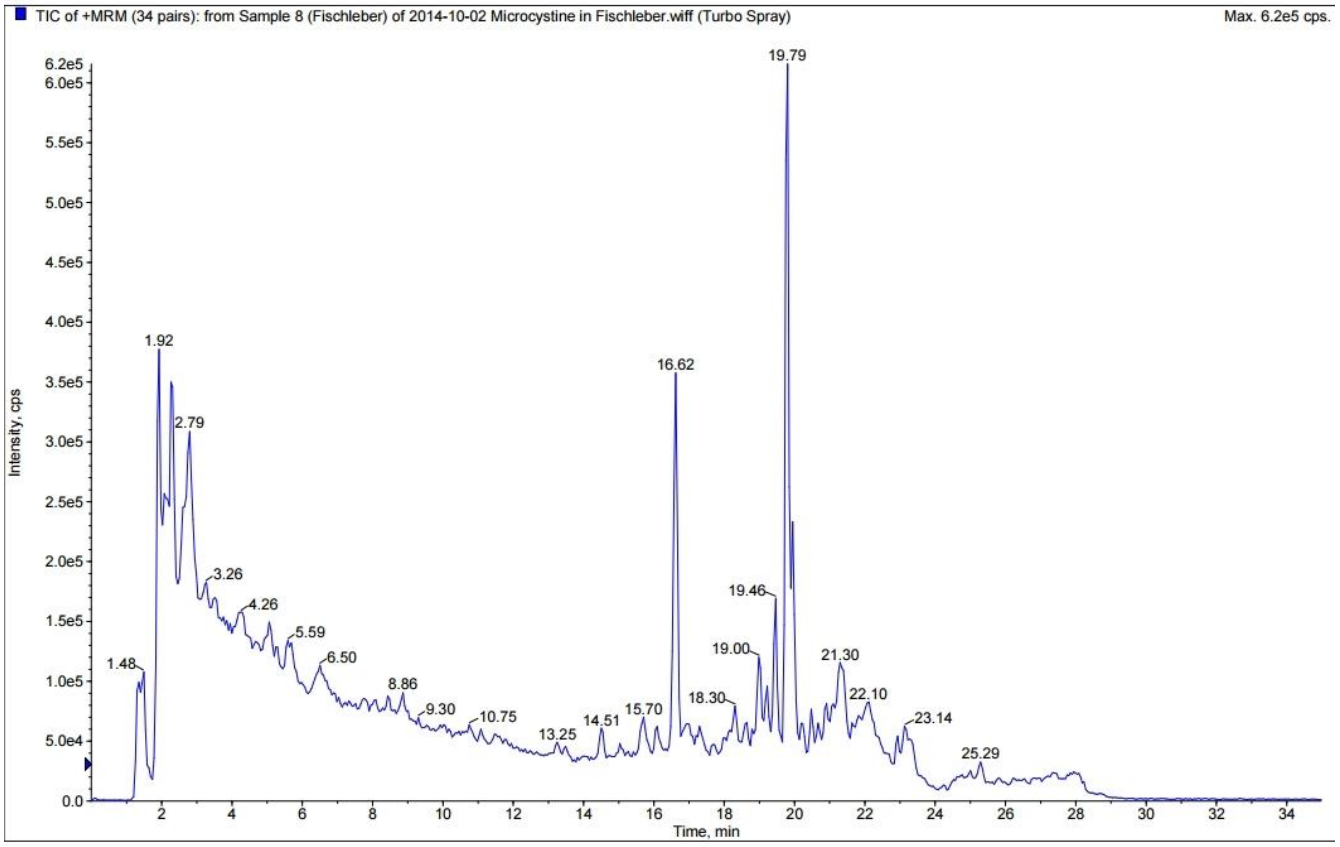
Reference standards of MC-RR, -LR, -YR, -LA, LF and –LW were purchased from Calbiochem/ Novabiochem (La Jolla, CA, USA). Acetonitrile and methanol obtained from VWR (Leuven, Belgium) were HPLC grade. All chemicals were at least analytical grade.

RESULTS AND DISCUSSION

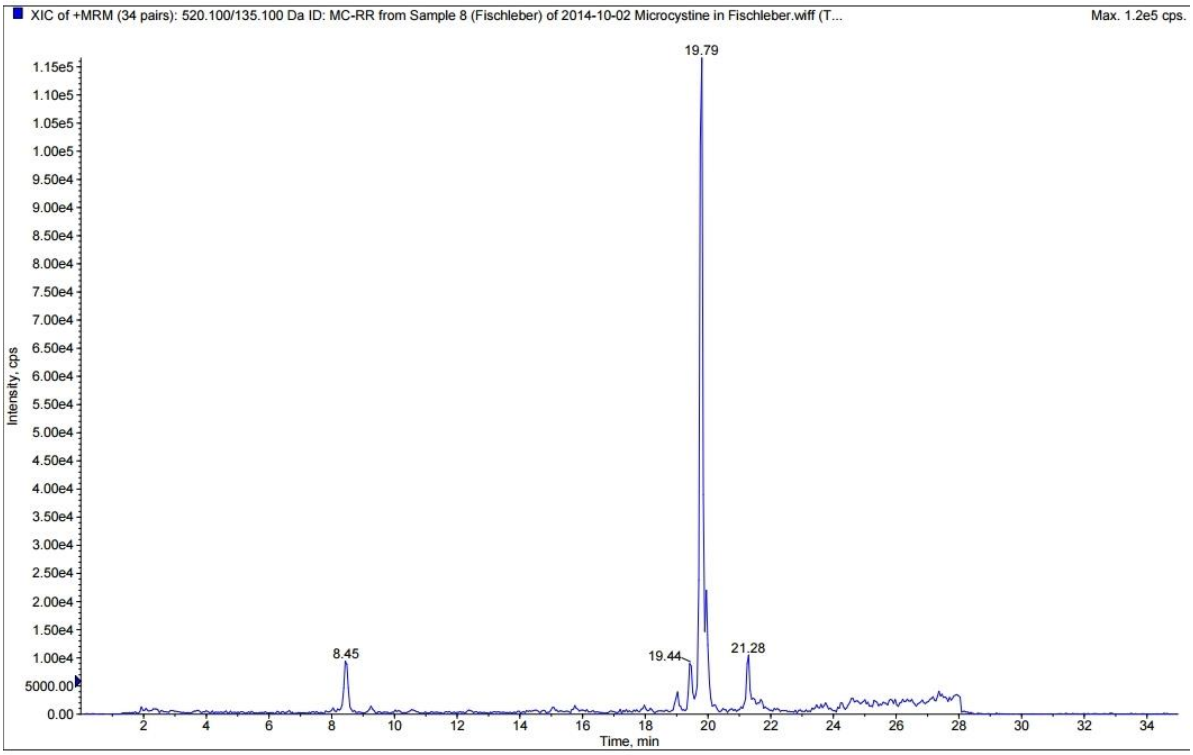
Nazira Bazar fish pond was covered with a heavy bloom of *M. aeruginosa* from February to September. During the bloom and fish collection period the water quality parameters were as follows: alkalinity 172mg/l, acidity 52mg/l, pH 9.4, hardness 160mg/l, carbon dioxide (CO_2)30mg/l, nitrite-nitrogen (NO_2-N) 0.01mg/l, phosphate phosphorus (PO_4-P) 0.78mg/l, ammonia (NH_3) >3.0 mg/l, dissolved oxygen (O_2) 4.8 mg/l at the surface, conductivity 940 µS/cm and temperature 30˚C (±2˚C).

Among the organs tested MCs were detected only from the liver of *O. niloticus*. No MCs was detected from the gut and muscle of the fish. Two variants of MCs were detected from the liver; MC-RR and MC-LF and their concentrations were 0.25µg/g and 0.22 µg/g, respectively (Fig. 1).

A. total ion count chromatogram of fish-liver extract



B. MRM chromatogram of MC-RR quantifier (retention time 8.5 min) in fish-liver extract



C. MRM chromatogram of MC-LF quantifier (retention time 19.2 min) in fish-liver extract

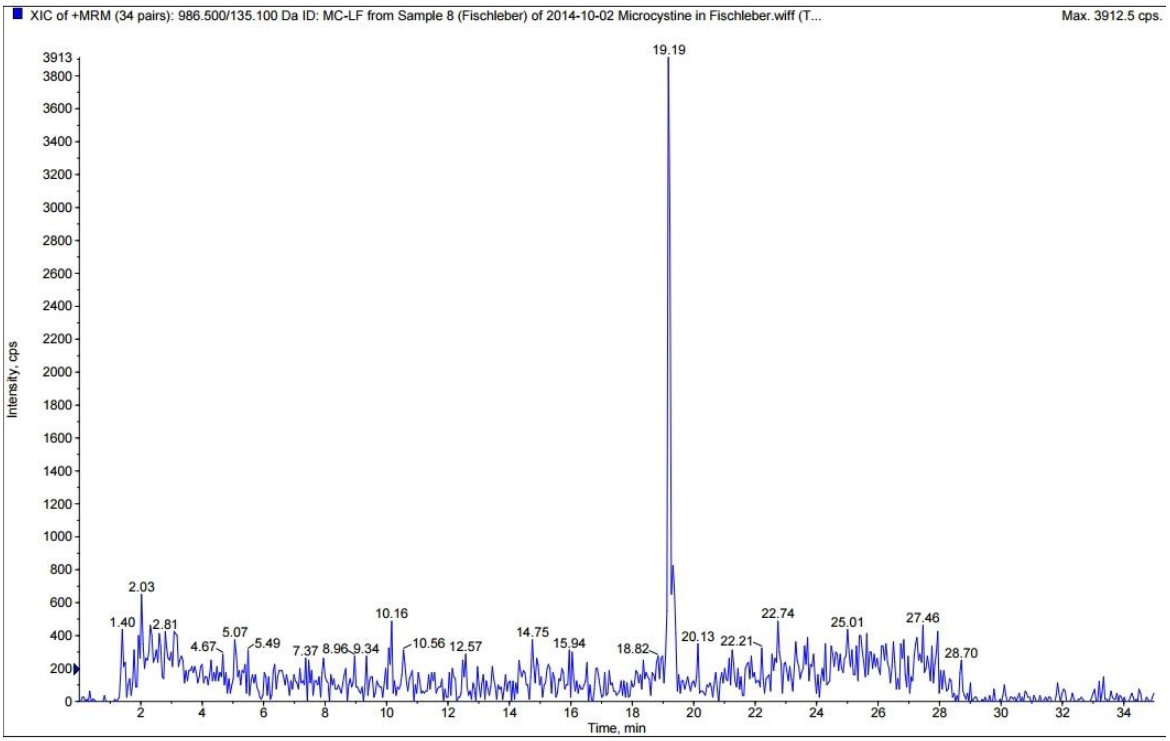


Figure 1. HPLC/MS-MS chromatogram of microcystins detected from liver extract of *O. niloticus.* A. Total ion count chromatogram B. MRM chromatogram of Microcystin-RR quantifier (retention time 8.5 min; [MC-RR+2H]2+ 520.1 > 135.0 C. MRM chromatogram of Microcystin-LF quantifier (retention time 19.2 min; [MC-LF+H]+ 986.5 > 135.0

MC-LR is the most common and highly toxic variant in the environment. Most toxicological experiments have been done for MC-LR and provisional tolerable daily intake TDI of 0.04µg/kg body weight per day has been established[25]. MC-LR, MC-RR, MC-YR and demethyleted variants of MC-LR and MC-RR are the most frequently detected MCs. MC-LF and MC-LW have been reported less often. But recent investigation showed that MC-LF and MC-LW are even more toxic than MC-LR (Table 2)[26]. Eriksson *et al.* (1990) and Fastner *et al.* (1995) reported that MC-RR (EC50 1500-4300 nM) is less susceptible than MC-LR (EC50 60-200 nM) in primary rat hepatocytes[27,28]. MC-RR is 5-37 times less toxic than MC-LR depending on the cell type (Table 1). Fisher *et al.* 2010 compared PP-inhabiting capabilities of MC-LR, MC-RR, MC-LW and MC-LF and stated that MC-LW and MC-LF produced cytological effects at lower equimolar concentration than MC-LR and MC-RR[29]. Additionally, MC-LW and MC–LF are more toxic than MC-LR and MC–RR in vivo and vitro experiment of OATP transfected HEK293 cells and the primary human hepatocytes.

Table 1. IC50, EC50 values of the investigated MC congeners[29].

	IC50(nM)		EC50 (nM)	
	PP1	PP2	Human hepatocytes (donor1)	HEK293-OATP1B1
MCLR	1.2	0.9	24.6	257.1
MCRR	1.5	0.9	900.2	1267
MCLW	1.9	1.1	0.4	4.0
MCLF	1.8	1.1	0.6	3.7

The higher toxicity is also reported for primary murine whole brain cells[30], CaCO-2 cells[31] (Table 2) and for the protozoa, *Tetrahymena pyriformis*[32]. Thus, risk assessments should include for the different MCs congeners rather than MR-LR alone. MC-LW and MC-LF have been reported from natural bloom of *M. aeruginosa* samples in Brazil[33] and Netherland[26]. The toxicity of MC-LW and MC-LF and its potential health hazard not yet been well established[34].

Table 2. Relative toxicity microcystin variants based on cellular alteration[30,31]

Cell type	Amount of toxins and exposure time	cytotoxicology	Microcystins			
			MC-LR	MC-RR	MC-LF	MC-LW
CaCO-2 (Compared to cell of small intestine)	50µM (44h)	Morphological change	No change	n.d	Clearly morphological alternations including apoptotic feature, shrinkage, bleeding, loss of cell contact	Less cellular effects
		Cell proliferation	7%	n.d	49%	70%
		Cell death	25%	n.d	36%	51%
mWBC (primary murine whole Brain cell)	5µM (48h)	Cytotoxic effect (reduction of cell viability)	54%	n.d	Complete loss of viable cell	33%

MCs can be accumulated through the food chain. Several authors have reported the accumulation of MCs, especially in aquatic invertebrates, which are essential elements of the diet of many different fishes[14,27]. The accumulation of MCs by tilapia in a natural environment has been reported by de Magathaes *et al.*, 2003 and Mohammed *et al.*, 2003[12,14]. In Bangladesh, tilapia has taken an important role in the commercial fish farming business and contributes 8.1% in fish production in inland water[22]. Tilapia culture has been promoted in small, seasonal ditches[35] because of rapid growth, good flavor, its high

resistance to poor water quality and its ability to convert the organic and domestic waste into high quality protein[36]. The present study is the first report on accumulation of MCs in fish tissue up to levels that pose a health risk for humans in Bangladesh. Total amount of MCs detected in the bloom was 73.14µg/l which is higher than WHO provisional guideline. Besides, a total 0.047 µg/g microcystins were detected in the liver sample. It should be noted that in Bangladesh especially in the rural areas people consume fish liver along with fish muscle. Recent studies have shown that MC-LF and MC-LW are more toxic than MC-LR[26]. The average portion of fish muscle eaten by a person is about 100-300g. If a man with a body weight of 80kg consumes100g of contaminated tissue (47µg MCs) would ingest 0.059µg/kg bodyweight of MCs-more than the TDI of 0.04 µg/kg body weight for MC-LR.

It was also observed that liver cells exposed with *M. aeruginosa* bloom were damaged both *in situ* and *ex-situ*[37]. Zhang *et al*., (2009) have reported substantial amounts of MCs in boiling water suggesting that eating soup of MC contaminated fish also poses a potential hazard to humans. MC-concentrations increased upon boiling probably due to the release of phosphate- bound microcystin[11]. Bioaccumulation of microcystins in the aquatic food web in Lake with potential risks to human health also been confirmed[38]. So, the proper regulation and a monitoring system should be developed for fish farms and household ponds in Bangladesh to prevent potential public health hazards.

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