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Accumulation status of microcystin-LR in cultured and natural samples of *Oreochromis niloticus* (Nile tilapia)

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Abstract
Toxic cyanobacteria *Microcystis aeruginosa* produce bio active compounds of Microcystin in water bodies. Serious health hazards were recorded in fauna and flora due to accumulation of MC-LR. Environmentally exposed *O. niloticus* random samples were collected from Beira Lake in mid of March; rich in scum. In vitro sampling was done separately using Beira Lake water. Water samples from the lake were added to the in vitro samples. The array of MC-LR accumulation was quantified using HPLC (High Performance Liquid Chromatography) and ELISA (Enzyme-Linked Immunosorbent Assay. MC-LR in edible fish parts were higher than the tolerable daily intake limit. Highest and the lowest ranged between 1.657±0.01 and 0.018±0.01 µg/kg $*10^3$. Boiling even at 100^0C did not degrade MC-LR. The mean bio accumulation factors ranged in between 1.103 and 0.006. The health risk assessed and the accumulation status predicted a positive relationship. Therefore, preventing from the intake of contaminated fish species would be much better for health.

Keywords: MC-LR, *Oreochromis niloticus*, bio-accumulation, bio accumulation factor

Introduction
Many of the water bodies are polluted and the pure nature is disturbed by the causes of nuisance algae. Eutrophication is a major issue which deteriorate the natural conditions of the rivers, lakes and reservoirs. This unbalance in environment of freshwater ecosystems favour the intense growth of cyanobacterial blooms [1, 2, 3]. These microbial colonies are mostly toxin producing cyanobacteria. Aquatic organisms are subjected to many health hazards and the mortality rate increases in excessive bloom conditions. The effects can be acute or chronic due to the toxin concentrations and the durations of exposure [1].
Microcystin is a cyclic heptapeptide molecule which has over 100 variants up to date. They generally differ in the nature of the two L-amino acids and in the degree of methyl substitution. Out of which the most toxic and the widely distributed is MC-LR. The most commonly observed species in the toxic cell colonies is *Microcystis aeruginosa*. Previous studies experimented in other countries such as Brazil, China and Egypt [4] state that *M. aeruginosa* has been the cause for human deaths caused by cyanotoxins. The toxicity affects occur as hepatotoxin, neurotoxin and nephrotoxins.

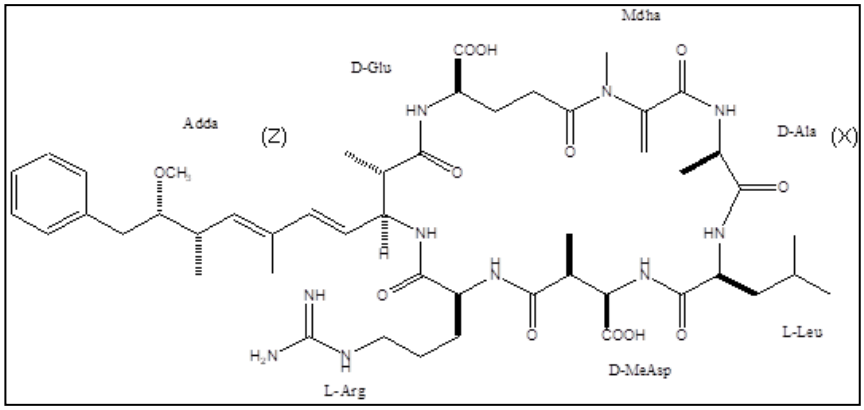

Fig 1: Structure of Microcystin-LR in which X and Z are variable of L amino acids [5]

Out of the several toxic cyanobacterial species, *Microcystis aeruginosa* is focused for the study due to the excessive and frequent formation of scums in water bodies of urban areas in Sri Lanka. Records state that Beira Lake which is situated in Colombo, is nourished with other cyanobacterial species such as *Lyngbya, Anabaena* and *Leptolyngbya* along with dominant and potentially toxic *M. aeruginosa* [6]. In the North Western province in Sri Lanka, many of the reservoirs are situated and the water ways are built in order to get sufficient water amounts for agricultural purposes. Moreover, the consumption of fresh water fish species from the contaminated environments has led to serious health problems. Specifically, *Oreochromis niloticus* being a Phyto planktivorous freshwater fish, the algal bloom consumption is relatively high. Since Sri-Lankan people consume Tilapia as a freshwater fish delicacy, the consumption of these can cause serious risk to the health. Tilapia is one of the top rated edible freshwater fish species which is consumed by most of the locale [7].

World Health Organization (WHO) concerning about the health risks to humans from contaminated drinking water, developed a guideline level for Microcystin-LR (variant of Microcystin) as 1 µg/l [8].

The toxins can be bio accumulated via aquatic food chains and affect top consumer levels containing vertebrates. Bio Accumulation Factor (BAF) is the quantification ratio between the biota and the environment at steady state equilibrium. It proves the idea to obtain the extent of risk assessed in order for safety precautions. Nevertheless, cyanotoxins have been a highlighted hypothesis for the prevalence of CKD (Chronic Kidney Disease) of unknown aetiology.

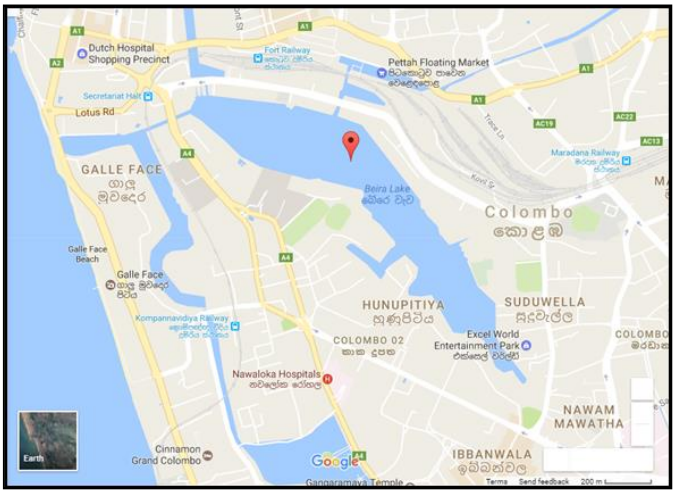

Fig 2: Location of Beira Lake

Present study focuses on assessment of the risk related with the MC-LR contamination and the bio accumulation in fresh water edible fish species; *Oreochromis niloticus* (Tilapia). The BAF gives a particular idea on how the toxins are magnified at the environment the biota lives in and the estimation of environmental health risk assessment.

Materials and Methods

Field studies

Beira Lake (6°55'46.59" N, 7°951'09.24" E) Eastern basin was selected to collect cyanobacteria (*M. aeruginosa*) bloom samples for the study. The period of collection was started in mid of March. *Oreochromis niloticus* (Nile Tilapia) were taken using cast net near by the north sluice. The fish were transported in polypropylene bags to the laboratory under proper conditions.

Laboratory experiment

In vitro experiment was carried out exposing fingerlings of *Oreochromis niloticus* to Beira lake bloom water samples. The fingerlings were obtained from the Dambulla Fish Breeding Centre. Before addition of the fish into the experimental and control tanks, they were acclimatized for a week by leaving out the water filled tanks in order to minimize the level of chlorine dissolved in water. The feed was provided daily according to the weights (1/5 of the Total weight).

Exposure to Microcystin

The Experiment tank with fingerlings was exposed to Beira Lake bloom water which contaminated with MC-LR ($1.25\pm0.18 \mu g ml^{-1}$) and the control tank was maintained only with fresh water. All the other conditions except microcystis bloom sample were kept the same for each tank.

Extraction and analysis of MC-LR from fish tissues

The fish were anesthetized by immersion in Tricane methane sulphonate (MS-222) solution for 5-10 mins before sacrificing [9]. The fish were kept on a dissecting tray according to standard methods [10]. The flesh, skin, head, liver and gills were separated and frozen (4 °C) until extraction of MC-LR. Then, all the samples were subjected to extract the MC-LR toxin. Methanol - Hexane extraction was employed according to Soares *et al*., 2004 [11]. In brief, the samples were crushed using the blender while adding 100% methanol in sample: methanol, 0.3g: 1ml ratio. The methanol added samples were put in the Shaker horizontally at 133 rpm for 30 minutes. Then, the samples were subjected to centrifugation at 6000 rpm for 15 minutes. The extraction procedure was triplicated for maximum extraction. The resulted supernatants from each fraction was pooled together. The methanol extract was then mixed well with an equal volume of 100% Hexane. It was added to bind lipid and then the mixture was (Methanol and Hexane) transferred into a separating funnel for remove lipid. Then the solutions were kept to settle for 30 minutes to ensure complete removal of the lipid. Thereafter, the extracted methanol sample was concentrated in rotary evaporator at 50° C. After concentration, the dry residue was dissolved in 1ml of methanol and filtered from 0.22µm Nylon filter. The filtrations were added to HPLC vials.

Quantification of toxin levels

Both tissue samples and water samples followed the same techniques. Bloom samples used to quantify after following the method modified based on Lawton *et al*. 1994 [12]. The quantification methods used for analysis were HPLC. Calibration was done with known concentrations of MC-LR. MC-LR recoveries were greater than 95% with a relative precision of 10%. The HPLC was used with sample injection volume of 25 µL into a 250 x 4.6 mm, C18 column at a flow rate of 0.8 ml^{min-1}. Two mobile phases were used for the gradient run (35% ACN/0.05% TFA and 65% Water/0.05% TFA).

ELISA method was used to quantify toxin of undetected samples by HPLC. Method was followed by Beacon Analytical SYSTEMS Inc. Microcystin plate kit (Catalogue number; 20-0068) according to manufacturer's instructions.

Bio Accumulation Factor

The calculation of bio accumulation factor was done according to the following equation [13].

BAF=kw/kb=CB/CW

C_B= Concentration of the toxin in the Biota

C_W= Concentration of the toxin in Water

Statistical analysis

Statistical analysis of differences between the data from the accumulation of toxin in different tissue parts was done using MINITAB 14.0 by One-way analysis of variance (ANOVA) and the normality test was done using Anderson-darling test. The p values less than 0.05 were considered to be statistically significant for all tests.

Results

The outcomes of the research have been tabulated below accordingly.

Table 1: The Toxin quantification of the water samples

Water Sample	Concentration in ppm (n=3)
Environmental	2.566±0.00
In vitro	1.248±0.18

The toxin concentration is higher in the natural environmental sample while the cultured sample showed lesser values. The natural environments give the ideal factors for the algal blooms to grow in excess.

Table 2: The maximum toxin concentrations in fish tissue samples

Sample Tissue Type	Mean Concentration detected /ppb	Weights/kg *10^{-3}	Calculated TDI µg/kg *10^3
Environmental samples			
Skin	2.610 ±0.01	6.3	1.657 ±0.01
Flesh	1.824 ±0.01	32.0	0.114 ±0.01
Head	3.689 ±0.01	49.5	0.224 ±0.01
Gills	1.914 ±0.01	2.4	0.798 ±0.01
Liver	1.981 ±0.01	0.3	6.603 ±0.01
Skin	2.498 ±0.01	3.0	0.833 ±0.01
In vitro samples			
Flesh	2.588 ±0.01	6.5	1.593 ±0.01
Head	0.274 ±0.01	15.4	0.018 ±0.01

*TDI is 0.04µg/kg bw/day [8] (If an average weight of an adult is 60 kg)

This method quantifies and proves that calculated TDI are relatively high in skin and flesh samples. Highest is recorded at liver samples. Gills record a moderate amount of toxin concentration. Gills are exposed to toxins moderately from the frequent uptake of oxygen via those. The head region recorded relatively very low amounts. Comparison with the tolerable daily intake (TDI) value, the calculated amounts show that they are much higher than the safe limit.

Table 3: Bio accumulation factor

Tissue Type	Mean Concentration of the Biota (µg/ml) *10^3	Water Concentration (µg/ml)	BAF *10^3
Environment sampling			
Skin	0.619	2.566	0.241
Flesh	0.132	2.566	0.051
Head	0.128	2.566	0.050
In vitro Sampling			
Skin	0.734	1.25	0.734
Flesh	1.103	1.25	1.103
Head	0.006	1.25	0.006

The above results show that the bio accumulation factor is higher in flesh and head. When considering the field sampling, the tissue type which records the highest toxin concentration is skin. The order fits as skin > flesh ≥ head. But the consideration of the in vitro sample can be stated as Flesh > Skin > Head.

Table 4: Health Risk Assessment

Sample	Minimum concentration/ µg/kg	Maximum concentration/ µg/kg	Max/Min/Equal than TDI (0.04 µg/kg)
Skin	0	1.23	Max
Flesh	0.01	0.08	Max
Head	0.01	0.17	Max

*Per capita fish consumption is 44.6g/day (Source: Ministry of Fisheries and Aquatic Resources Development, 2015)

*TDI is 0.04µg/kg bw/day [8] (Average weight of an adult is 60 kg)

All the results in table 4 above show that the values have exceeded the recommended limit proposed by WHO.

Discussion

Contamination of MC-LR in vertebrates as well as in invertebrates has been addressed in recent studies [14]. In Sri Lanka, it has no records of determining fish bio accumulation effects so far. Around the world, there are occasions which prove that cichlidae family mainly; *Oreochromis niloticus* are considered as bio indicators. Aquatic organisms are found to be developed with tolerance to toxification more than mammals. It is proved with evolutionary history. The analysis for consumable parts have not been done so far in Sri Lanka. Therefore, thorough evaluation is needed. Mainly the effect on liver has been researched in depth because MC-LR is a potent hepatotoxin. Besides that, gills and kidney are assessed and documented. For examples, Rainbow trout (*Onchoryhchus mykiss*), silver carp (*Hypophthalmichthys molitrix*) and common carp (*Cyprinus carpio*) have been extensively used to identify the physiological, chemical and genetical alterations that cause due to the Microcystin intoxication [15]. The invertebrate studies have been carried out majorly using clams and fresh water snails [16].

Present study was aimed to identify and verify the highest accumulation of Microcystin in various edible fish tissues. Microcystin exposure was done by providing the natural environment at maximum possible ways. The growth of *Microcystis* bloom varied with the environmental conditions

[17]. To get a clear picture, the growth of the cyanobacterial bloom monitoring is essential. The intensity of the scum increases during the stationary phase producing the maximum possible growth and this has been the best period to collect the bloom from the natural site [18].

In this study, the selected site was Beira Lake, Colombo because it is rich in all the effluents and pollutants causing a high nitrogen and phosphate load. Apart from that, the location is optimum for the growth of algae with the wind direction and the ample sunlight.

During this study, the risk assessment was done. Due to many recorded serious health issues posed by the unnecessary consumption of contaminated food and water, it is given higher consideration to conduct this study. The hepatotoxic MC-LR affects the liver as the main targeting organ causing hepato-carcinoma and cellular disruptions [19]. Many of the recent studies have focused on the acute and chronic health effects that can cause death to human and other animal species. Vertebrates as well as the invertebrates are undergoing major causes as the population decreases. The ecological niches and the conditions with optimized factors are destroyed due to the scum formation and ingestion; accidentally.

The presence and accumulation of MCs in different fish tissues have been reported by experimental and field studies. There are several reports on accumulation of MCs in liver [4, 20], gills [21], intestine [4], kidney [22, 16], muscle [11] and brain [5]. The study on liver and gills recorded 6.603±0.01 and 0.798±0.00 respectively (Table 2) underlining the previous studies.

The finding of Chen *et al*. [24] has a harmonizing nature with the results of the present study in accordance with flesh samples experimented at in vitro conditions. The activation of the detoxifying mechanisms forming conjugations with MC-LR has been a major possibility and a basement for the observed results. A better understanding on the biotransformation process is necessary to improve the knowledge on mechanisms involved in toxicity of MCs, and on the response of different organs to such toxins, considering that different organs react in different ways, or with different intensity, to the presence of a toxin. Additionally, the exposure route could also influence the uptake and severity of effects.

Whilst considering about the lipid contents of flesh, head and skin, over time the fat composition increases with body weight. And that phenomenon makes the fundamental baseline to activate detoxification as prior defence mechanism. The suggestion is solidified by the studies of Cazenave *et al*., (2006) [21] and Malbrouck *et al*., (2003) [20].

The present study shows that at natural conditions such as optimum environments can be specific ecological niches, where fish can migrate from one place to another. The Bio Accumulation Factor (BAF) gives the values for the biota and the water concentration when the compositions are in steady state equilibrium. Previous studies have stated that BAF as an indicator for the presence and risk of contamination and accumulation of MC-LR along the trophic transfer phenomenon. Adamvosky *et al*., [15] stated that the BAF values range in a way that it recorded the slightly higher levels in the liver of common carp in comparison to those in the liver of silver carp. Calculated bio concentration factors ranged from 0.051 to 1.103 in the muscle/flesh, from 0.241 to 0.731 in the skin and in head from 0.006 to 0.050 when considering both samples. As per the knowledge up to date, the BAFs for MCs in fish were not previously reported extensively for a proper comparison. The study states that the skin and flesh accumulate high amounts of toxin concentration when exposed to continuous cyanobacteria blooms. Sensitive values have been obtained at the present study in skin and flesh, suggesting that risk assessed is higher at consumption of those edible parts. It is with higher health risk if toxin contaminated fish are consumed and it is mandatory to assess the situations in water ways to avoid higher algal blooms that may cause harm to both flora and fauna.

Conclusion

The rising nutrient loads due day to day anthropogenic activities lead to high amounts of algal bloom formation. These can lead to dire impacts on both flora and fauna as a whole. Present study proved that the palatable tissues of Nile Tilapia are highly intoxicated due to MC-LR. The highest amounts of the toxin accumulated in skin and moreover, it is recommendable to avoid that in the fish intake. Furthermore, the flesh which is the highest consumable part is seen to be more prone to the accumulation of the toxin over time. This can be a turning point in the aquaculture in areas with contaminated waters. The culturing should be done under regular monitoring of water quality and the other important aspects. The head of the fish species are remained to be not harmful nevertheless, it is also contaminated with the slightest amounts; less than TDI values. However, the detoxification mechanisms inside the organism's body plays a major role in scavenging the toxin radicals and molecules. If the monitoring can be aligned with WHO guidelines and the awareness among the public can reduce the potential health hazards.

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