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FREE MICROCYSTIN-LR AND ITS BIOACCUMULATION IN SOME FISH TISSUES, COLLECTED FROM SAGAR LAKE.

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ABSTRACT

Microcystis aeruginosa sp. (a Cyanobacterium) synthesizes the cyclic heptapeptide Microcystin-LR, a potent toxin toward humans and other animals. MC-LR is one of the most potent type of isoform in nature with their potent hepatotoxicity and all the Microcystins are the potent inhibitors of serine/ threonine protein phosphatase 1 and 2A. Fish are naturally exposed to MC through ingestion of contaminated food and through dissolved toxins. In the present study MC-LR bioaccumulation, its distribution in the some tissues of Sagar Lake fishes has been reported. The MC-LR bioaccumulation was examined in the following fish *Puntius ticto*, *Puntius conchonious*, *Glossogobius giuris* and *Oreochromis mossambicus c*ollected from the Sagar Lake. Free form extractable MC-LR in fish tissue samples were examined by High Performance Liquid Chromatography with a Photodiode Array Detector (HPLC-PDA). The mean concentrations of MC-LR bioaccumulation observed in the muscles and the liver, significantly difference among all fish species in which muscles shows the highest accumulation of the toxins. That fish species are commercially important from the lake and they accumulate toxic MCs in tissues relevant to human consumption. The average daily intake (ADI) of MCs in the muscles of all fishes exceeded the human tolerable daily intake (TDI) 0.4µg/kg body weight per day set by World Health Organization (WHO).

Keywords: *Microcystis aeruginosa*, Microcystin-LR, Freshwater Fishes, Bioaccumulation.

1. INTRODUCTION

In addition to the implication of being widespread in nature often bloom farming, various cyanobacterial species share equal ability to produce toxin. In cyanotoxins Microcystins (MCs), are the most abundant in eutrophic water, are cyclic heptapeptides with severe hepatotoxic action. Microcystins are a family of monocyclic heptapeptides produced by brackish and fresh water or cyanobacterial bloom of the genera *Microcystis* (Order: Chroococales), *Oscillatoria* (Order: Oscillatoriales), *Dolichospermum* (Order: Nostocales), *planktothrix*(Order: Oscillatoriales), *Pseudoanabaena*(Order: Synechococcales) and *Woronichinia* (Order: Chroococales) (Pearl and Otten, 2013; Dietrich and Hoeger, 2005).

To date over 100 MCs variants are known, the variations are based on the two variable L-amino acids that lead to the main structure of any isoform: Microcystin-LR (MC-LR), MC-RR and MC-YR (Zastepa et al,. 2005). Microcystins are composed of seven amino acids (WHO, 1991; EPA, 2015). (Fig.1). Among them the unusal type of Adda (2S, 3S, 8S, 9S) -3 amino acid-9- methoxy-

2,6,8- trimethyl-10-Phenyldeca, 4,8-dianoic acid. (hydrophobic & aromatic) often linked with the toxicity of the molecule, that interact to the hydrophilic grove of the Seriene/ threonine Protein phosphatases (PPs)(Ruangyuttikarn *et al.,* 2004). Chemically & physically Microcystins are very stable (Janes and Orr, 1994).

Microcystin- Leucine on position 2 and Arginine on Protein 4 cought to abbrebriated as MC-LR, chemical formula is $\mathbf{C_{49}H_{74}N_{10}O_{12}}$. It is solid in physical state, molecular weight about 994 Dalton and density 1.29 g/cm^3. Because of hydrophilic in nature it is highly soluble in water; it is also soluble in ethanol and methanol. MC-LR is the most toxic variant of Microcystin and these days most concerned and most diverse area in research (Li *et al.,* 2008). MC-LR is hydrophilic in nature and generally it directly cannot penetrate cell membrane of vertebrate, therefore needs ATP transporter for their uptake (Von Apeldoorn *et al.,* 2007). MC-LR is the strongest inhibitor of Serine threonine protein phosphatase 1, 2A, 2B and act at tumor promoter, hyperphosphorylation occurs as resultant of PKs activity and followed by disruption of the liver's cytoskeletal, micro-filament, intermediate filament and microtubules. (Wiegand and Pffumacher, 2005).

MCs could be responsible to trigger the formation of excess reactive oxygen species (ROS), which results in protein oxidation, lipid peroxidation (LPO) and DNA damage (Cadenas, 1989; Amado and Monserrat, 2010; Sun *et al.,* 2012; Qian *et al.,* 2014). MCs were detected in various organs in animals, such as muscle, kidney, liver and brain. MCs can trigger acute toxicity in these organs such as in form of liver dysfunction and hemorrhage (Vander Merwe *et al.,* 2012). Briefly, an unidentified multi-specific Organic anion transporter (Oat) or bile anion transporter is responsible to carries MC-LR into the organs such as liver.

In mammals MC-LR via the oral route, it directly transported across the small intestine (ileum) into the blood stream through the bile acid transporters, the system present in the hepatocytes and the cell lining of the small intestine and its concentration in liver likely as a resultant active uptake by hepatocytes (WHO, 1998). The event in liver leads to uncertain type of massive pooling of blood followed by destruction of sinusoid and eventually it caused death by hemodynamic shock (Mackintosh *et al.,* 1990; Ott and Charmichael., 2006).

The world health organization (1998) has proposed a tolerable daily intake (TDI) of MC-LR to reduce risk as 0.04µg/kg body weight per day and in drinking water 1.0 µg/L as a maximum allowable concentration of this toxin (free and cell bound). Long term exposure under toxin must be considerable. Microcystins are relatively very stable and resistant molecule against biological, physiochemical factors including sunlight, temperature and enzyme. In aquatic nature these toxins can be easily absorbed by aquatic organisms including fish, invertebrates and aquatic plants (Jiang *et al.,* 2011; Galanti *et al.,* 2013; Khalloufi *et al.,* 2013). Microcystins are synthesized non-ribosomally by the help microcystin synthetase complex (Kaebernick and Neilan, 2001) and the whole process of their synthesis is energy (ATP) dependent type.

2. MATERIAL & METHOD

2.1. SAMPLING SITE

Sample was collected from Sagar Lake (Madhya Pradesh, India). Sagar city falls a few kilometres to the North of tropic of cancer at an altitude of 517m and at the latitude of 23°50' N and longitude of 78°45' E. The lake popularly known as "Lakha Banjara" is divided into two

parts, the main lake with spread area of 1.1848 ha. and small lake with water spread area of 0.4046 ha. The catchment area of the lake basin is 1817 hectare out of which the total water spread area is 145 ha. After two years of extensive study on Sagar Lake, there are total 21 species of fresh water fishes belonging to 6 order, 11 families and 17 genera have been recorded. In them most abundant types of species are *Puntius ticto, Puntius conchonius, Puntius sophore and Rasbora rasbora* (Family: Cyprinidae), *Glossogobius giuris* (Family: Gobidae), *Oreochromis mossambicus* (Family: Chichlidae) and *Lapidocephalichythys guntia* (Family: Cobitidae) etc (Wani and Gupta, 2015, Table 1).

Moreover, the inflow or runoff of costal water from the industry, domestic and non point sources are the main factors of water pollution in the Lake situated within the city. Now a days eutrophication of Lake is the major issue with the regular occurrences of cyanobacterial surface blooms in the summer season every year. Sampling site was selected at location where the *Microcystis aeruginosa* bloom flourished the lake whole year (Figure.2).

2.2. BLOOM AND FISH SAMPLE COLLECTION AND PREPRATION

Samples were collected from the Lakha Banjara, Sagar Lake in the month of June, July, August, September and October 2015. When collected from Lake the water bloom sample was most dominated with *Microcystis* in summer season. Fishes were captured by fisherman with the help of fishing net for the determination of MC-LR concentration in liver and muscle. All the samples were placed on ice and transported immediately to the laboratory.

In Sagar, Lakha Banjara Lake all these fishes are the most abundant species, found equally distributed. Threat status of these fishes was based on the survey report on Conservation Assessment and Management plan (CAMP) for fresh water India (Molur and walker, 1998) and IUCN, 2014.

Taxonomical rank	**Scientific name**	**Species richness**	**IUCN status**	**CAMP status**
1. Order- **Cypriniformes**				
Family Cyprinidae	*Puntius ticto*	+++	LC	LR-nt
	Puntius conchonius	+++	LC	LR-nt
2. Order- **Perciformes**				
Family- Gobiidae	*Glossogobius giuris*	+++	LC	LR-nt
Family- Cichlidae	*Oreochromis mossambicus*	+++	NT	NE

+++= Abundant (71-100 % of total catch), LC= Least concerned, NT= Near Threatened, LR- nt= Lower risk near Threatened, NE= Not evaluate.

Table: 1. Fish species collected from Sagar Lake, their abundance and conservation status in Lake (Wani and Gupta, 2015).

2.2.1. EXTRACTION OF FREE MC-LR FROM BLOOM SAMPLE

The process of isolation of toxins was carried out as described by Harada *et al.,* (1988). Total 10 grams of dried cells was extracted with 100 ml of methanol and water (70:30), the samples were sonicated three times for 5 min. Total extract was frozen overnight and then centrifuged. Supernatant collected and this process repeated 3 times for pooled extraction of MC-LR. The filtrate was concentrated on Octadecil–Silane cartilage (C18), which was washed with 20 ml of

distilled water, followed by 20 ml of 20% methanol and eluted with 20 ml of 100% methanol. The toxin containing supernatant was completely evaporated to dryness.

2.2.2. EXTRACTION OF FREE EXTRACTABLE MC-LR FROM FISH TISSUE

Total 3 omnivorous fish species (*Puntius ticto, Puntius conchonius* and *Oreochromis mossambicus*) and 1 carnivorous species (*Glossogobius giuris*) were collected monthly from 6 individual of total 4 fish species. In this manner to determination of MC-LR in fish tissue (Muscle and Liver) were dissected immediately, and the liver and muscle were separated 20-40 gm respectively and weighed, the muscle and liver were removed and washed separately with distilled water to avoid cross contamination. All the tissue lyophilized for 72 h. The individually 5 gm tissue of each fish homogenized with water: Butanol: methanol (MeOH) (1:4:15) v/v in homogenizer for 10 mins. Samples were centrifuged (25000×g) for 20 min. The supernatant was transferred in a clean glass flask. The remaining residue was re-extracted with the same solvent composition as taken. The extract was evaporated at 40^0 C to dryness with the help of rota-evaporator and re-dissolved in MeOH. An aliquot of 20 µl was injected onto HPLC system for quantification.

2.2.3. TOXIN ANALYSIS BY HIGH PERORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The MC-LR present in the crude extract were quantified by analytical High Performance Liquid Chromatography (HPLC) following a modified version of HPLC using Waters Alliance (555) equipped with a photodiode array (PDA) detector 2998. The analysis of Microcystin-LR was performed using are reversed phase column (LiChrosphers 100RP-18 end- capped, 25cm×4.6 mm,5 µm, Sigma Chemicals, and kept at room temperature. The mobile phase was of isocratic run of (A) MeOH and (B) Mili Q ultrapure H2O (70:30) with a flow rate of 1 mL/min for 20 min, injected volume of sample was 20 µL and the PDA range was 210–400 nm with a fixed wavelength at 238 nm. The MC-LR spectrum and retention time of the samples were compared with a standard MC-LR (Sigma- Aldrich, 95% purity). All the solvents were used of HPLC grade quality, filtered in a 0.2 µm hydrophilic polypropylene membrane filters and degassed before use.

3. RESULT AND DISCUSSION

The chromatogram of the MC-LR standard and the extract of *M. aeruginosa* are compared (Figure.4). It is clear that the toxins were taken up and a part of toxin was extractable. During the study period, there were great variations in the MC-LR concentration in various organs of the fishes.

3.1. BLOOM SAMPLE OF *Microcystis aeruginosa*

The bloom samples were analyzed for the identification by the help of light microscope. Colonies of bloom were identified as *M. aeruginosa* with cell having diameter of 3 to 5 µm, spherical in shape. Determination MC-LR from *M. aeruginosa* bloom observed by the help of High performance liquid chromatography technique (HPLC), collected from Sagar Lake, in year 2015, contained MC-LR at 20 µg/g of DW (Figure. 4(2). RT: 2.923 and Area: 6288)

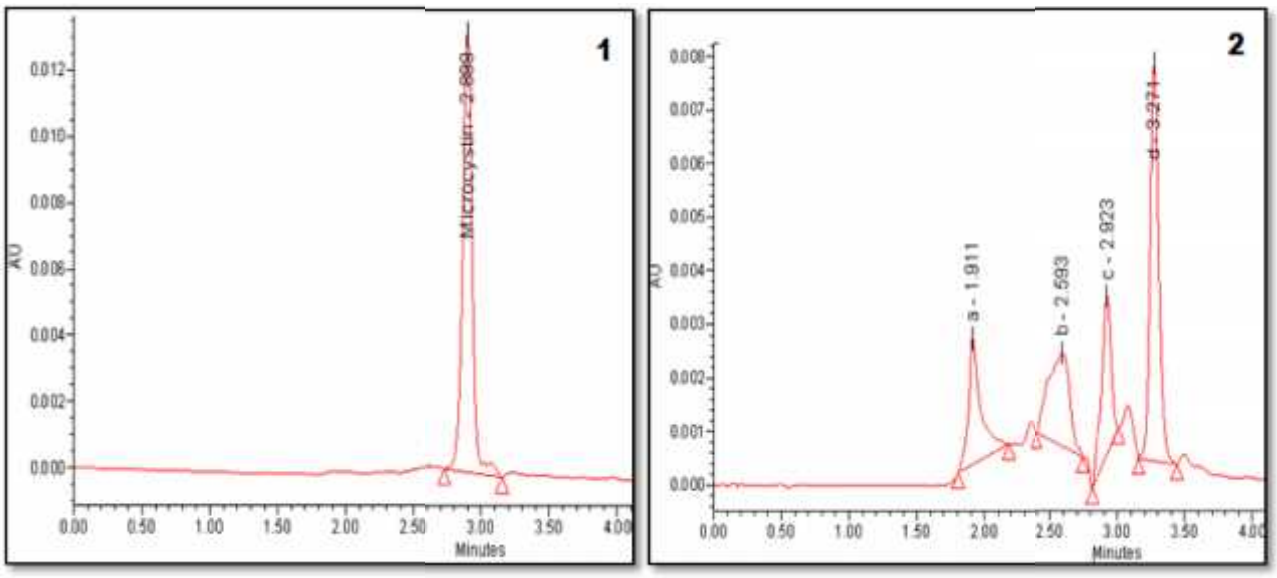
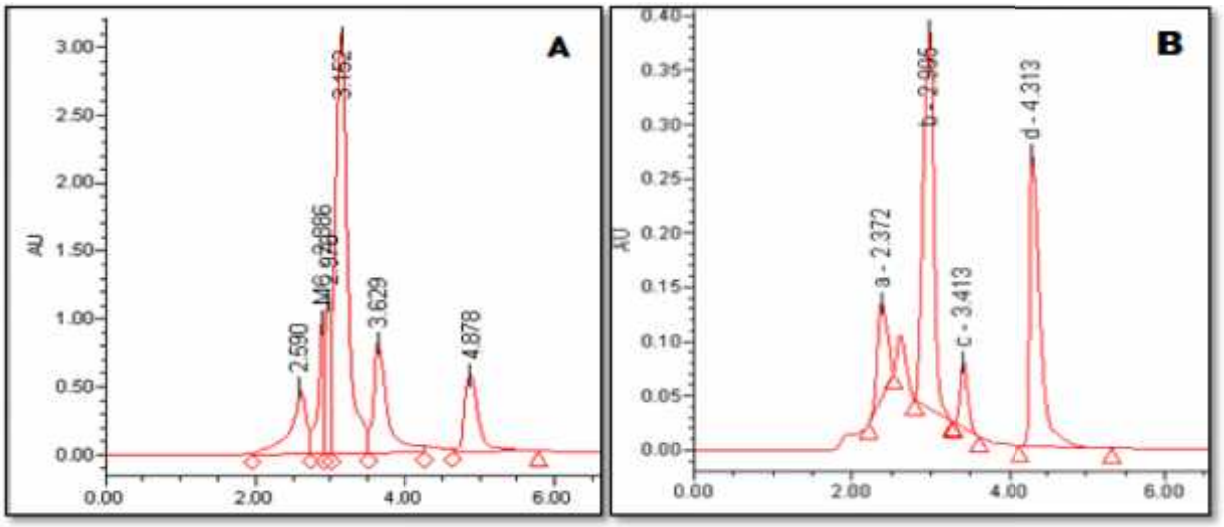


Figure.4. HPLC chromatograms (1) Microcystin-LR standard; (2) Microcystin extract containing MC-LR in *Microcystis aeruginosa* bloom collected from Lakha Banjara, Sagar Lake. Spectrum determined at PDA 238 nm. The sample injection volume was 20 µl and the retention time of Microcystin-LR standard was around 2.9.

3.2. ACCUMULATION OF MC-LR IN FISH

In this study total no. of 70 fishes were caught for the bioaccumulation of toxin, in 5 month (June, July, August, September and October) and the liver and muscles samples from the 4 fish species were analyzed. Fisher and Dietrich (2000) informed that cyanobacterial digestion specifically dependent on their feeding habit of fish such as Cichlids and Cyprinids, with planktivorous, herbivorous and omnivorous feeding habits having the wide digestive surface area than carnivorous fish. Hence exposure under MCs being more likely, on the other hand carnivorous fishes could retain MCs not only directly from planktivorous prey but also from after consumption of large zooplankton and other invertebrates (Wilson *et al.*, 2008).



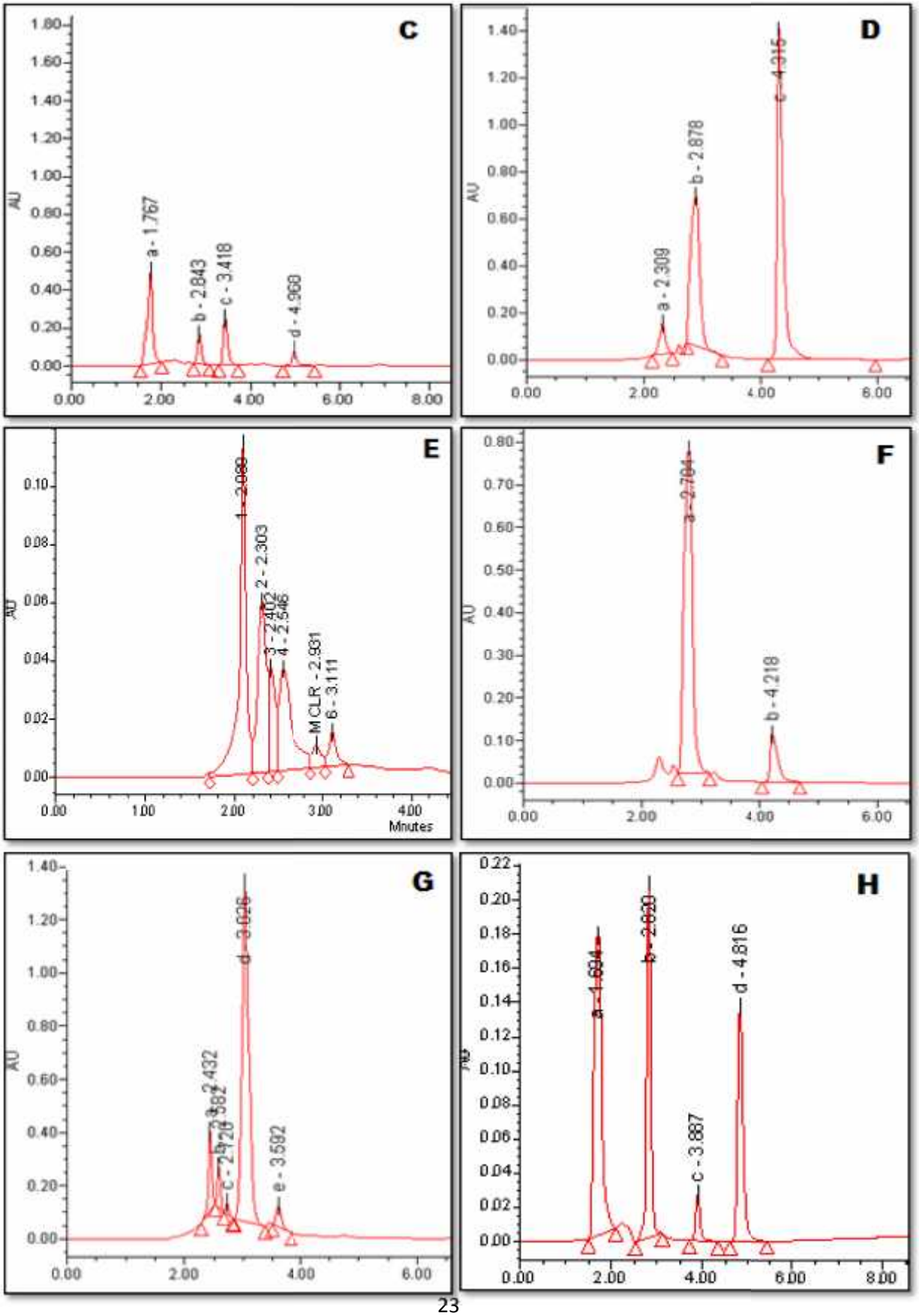

C
a - 1.767
b - 2.843
c - 3.418
d - 4.968
D
a - 2.309
b - 2.878
c - 4.315
E
1 - 2.069
2 - 2.303
3 - 2.402
4 - 2.546
M CLR - 2.931
6 - 3.111
Minutes
F
a - 2.704
b - 4.218
G
d - 3.026
2.432
e - 3.592
H
a - 1.694
b - 2.620
c - 3.887
d - 4.816
AU

Figure.5. HPLC chromatograms of (A) *Puntius ticto* (Liver), RT: 2.886 and Area: 5632 (B) *P. ticto* (Muscle), RT: 2.905 and Area: 7236 (C) *Gobius* sp. (Liver) RT: 2.843 and Area: 5452 (D) *Gobius* sp. (Muscle) RT: 2.878 and Area: 6020 (E) *Puntius conchonius* (Liver) RT: 2.931 and Area: 5524 (F) P. *conchonius* (Muscle) RT: N/D (G) *Tilapia* sp. (Liver) RT: 2.920 and Area: 4860 (H) *Tilapia* sp. (Muscle) RT: 2.820 and Area: 5620.

S.N.	Fish	Feeding habit	Sample	MC-LR Concentration (µg)
1.	*Puntius ticto*	Omnivorous	Liver	0.66 µg/g FW
			Muscle	1.45 µg/g FW
2.	*Glossogobius giuris*	Carnivorous	Liver	0.76 µg/g FW
			Muscle	1.98 µg/g FW
3.	*Puntius chonconius*	Omnivorous	Liver	0.58 µg/g FW
			Muscle	N/D
4.	*Oreochromis mossambicus*	Omnivorous	Liver	0.46 µg/g FW
			Muscle	0.62 µg/g FW

Table: 2. MC-LR concentration (µg/g FW) based on HPLC chromatogram in fish muscle and liver tissue.

Several authors reported Microcystin concentrations in zooplankton, shellfish and fish (Vasconcelos., 1995; Williams *et al.,* 1997; Tencella *et al.,* 1994). Briefly bioaccumulation is the uptake and retention of any compound or chemical by an organism from any environmental sources (food, water etc.) EPA(2000b). EPA (2003) has proposed that total bioaccumulation factor as the ratio of the concentration of a compound or chemical in the tissue (for example muscle) of an aquatic organism to its concentration in water (free dissolved plus cellular).
Toxin calculated upto 0.66 µg/g to 0.76 µg/g in liver and in muscle it varies from 0.62 µg/g to 1.98 µg/g fresh weight in studied fishes, as determined by the help of High performance liquid chromatography (HPLC).

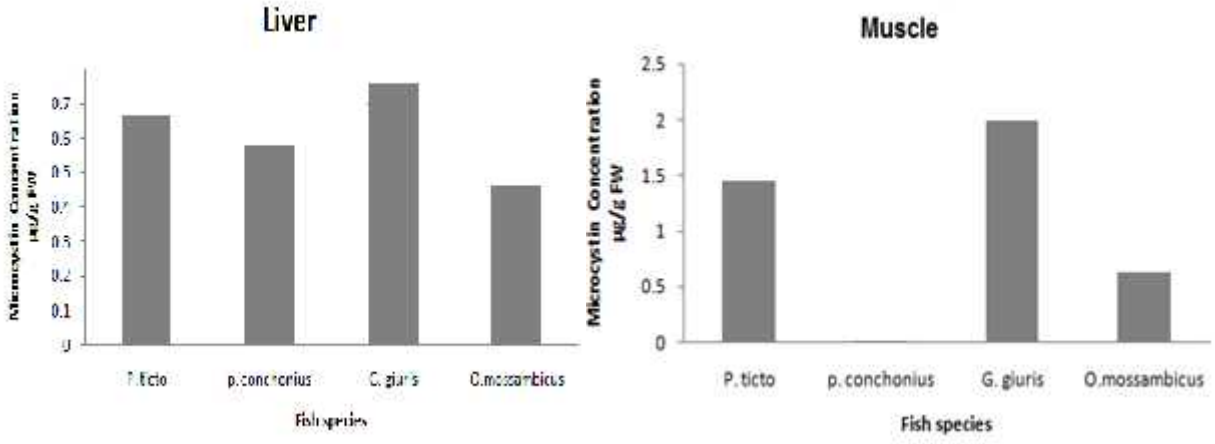


Figure.6. Microcystin-LR (MC-LR) content in the fishes determined by HPLC shows MC-LR concentrations accumulated in fishes.

The results of the study are summarised and shown in the table 2 and Figures5and 6. In the present study, MC-LR content in the muscle and liver was highest in carnivorous fish followed by omnivorous fish, that indicating the MCs showed the general tendency to ccumulate up to the chain in the Sagar lake. Figure 5 shows the MC-LR content in different organs of the Fish tissue (liver and muscle) sample. In the liver, MC-LR was detected in *Puntius ticto, P. conchonius, Glossogobius giuris* and *Oreochromis mossambicus,* reaching 0.66 µg/g, 0.58 µg/g, 0.76 µg/g and 0.46 µg/g FW, and in muscle MC-LR was 1.45 µg/g, Not detected, 1.98 µg/g and 0.62 µg/g FW respectively. No MCs were detected in the *Puntius conchonius* muscles contents.

Fishes are typical inhabitants of the aquatic environment which conspicuously exposed under MCs. Fishes can absorb cyanobacterial toxins either directly through water or may consume up through food. Microcystins (MCs) can affect mainly the liver, gill, kidney (Gupta and Guha., 2006). To date, various laboratory experiments and field studies have been conducted to investigate the MCs bioaccumulation in a different-different type of aquatic organisms (Xie *et al.,* 2007; Yokoyama and Park, 2002; Chen *et al.,* 2006; Magalhães *et al.,* 2003; Song *et al.,* 2007; Jia *et al.,* 2014) and etc.In natural environment, MCs can accumulate freely in a wide range of aquatic biota such as fish (Deblois *et al.,* 2011; Bieczynski*et al.,* 2014), crustaceans (Pinho *et al.,* 2005; Galanti *et al.,* 2013; Sabatini *et al.,* 2015), gastropods (Zhang *et al.,* 2012c; Lance *et al.,*2010), mollusks (Sabatini *et al.,* 2011) and macrophytes (Zhang *et al.,* 2011b; Romero-Oliva *et al.,* 2015).

However it is important to understand how the bioaccumulations are reported. Many studies have used a brief definition of bioaccumulation to mean that toxins present constitutively in organism, had not referred main concept of bioaccumulation factor (concentration of toxin in tissue divided by available toxin). Xie *et al.,* (2005) studied on the MCs bioaccumulation in freshwater fishes of Chaohu Lake in China at different trophic levels from the shallow, large and eutrophic Lake. Resultant MCs content in the muscle and liver of carnivorous fish *(C. ilishaeformis*) were 2.22 and 5 µg/g D.W and in phyto-planktivorous fish Silver carp (*H. molitrix*) were 1.65 and 1 µg/g D.W, respectively.

The mean level of MC-LR was higher in pond or stagnant water as compared to Lakes. As a planktivorous fish, *Tilapia* (*Oreochromis*) consumes cyanobacteria mainly *Microcystis* and other filamentous species dominant at the pond. The lake is very important source of raw water for agriculture, domestic and drinking purpose as well as for livelihood and food source of local fishermen.

Toxicity of lake water or eutrophic environment of lake causes extinction of flora and fauna of lake. These type of toxins imbalance the food web of aquatic environment. The presence of these toxin variants in lake water may affect fish growth and their productivity. The World Health Organization (WHO) has determined a tolerable daily intake value (TDI) of 0.04 µg kg/ BW per day for MC-LR (Chorus and Bartram,1999). The average portion of fish eaten by a person is about 100–200 gm, and therefore a 100-gm portion would contain 2.64–49.7 µg of MC-LR equivalent, or about 1.3–25 times the recommended value TDI for MC-LR. On the other hand, because MCs are heat stable, they are not broken down by cooking (Harada *et al.,* 1996).

4. CONCLUSION

In the present study MC-LR concentration were quantified in four fish species and also in *M. aeruginosa* bloom sample collected from Lakha Banjara Lake, Sagar. During study spatial

variation were determined in fish tissues and bloom sample. The concluded remark of this study reveals the consumption of fishes collected from this Lake is not safe for human consumption. Resultant of this study MC-LR concentration is higher in the muscle of Tilapia than the other fish species selected in the study, while *Puntius ticto* and *Puntius conchonius* accumulated high MC-LR in liver than the muscle. The difference is not significant. Conspicuously biotic and abiotic factors and consumption of algal cells influence the accumulation of MCs in fishes. Further more studies needed to determine the cyanotoxin concentration in aquatic bodies in Sagar Lake in order to evaluate the inappropriate health risk of human consumption.

5. ACKNOWLEDGEMENT

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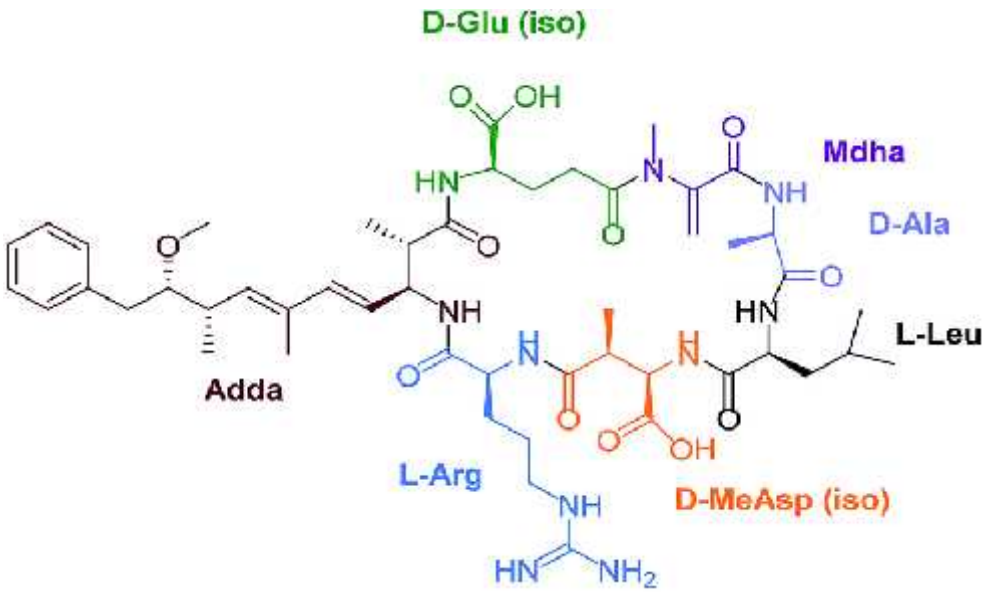

Figure.1: General structure of Microcystin (McElhiney & Lawton., 2005)

Figure.2: (1) Satellite View of Sampling Site (2) *Microcystis aeruginosa* bloom of the Lakha Banjara Lake, Sagar (Madhya Pradesh), India. (Photographed by Pinki).

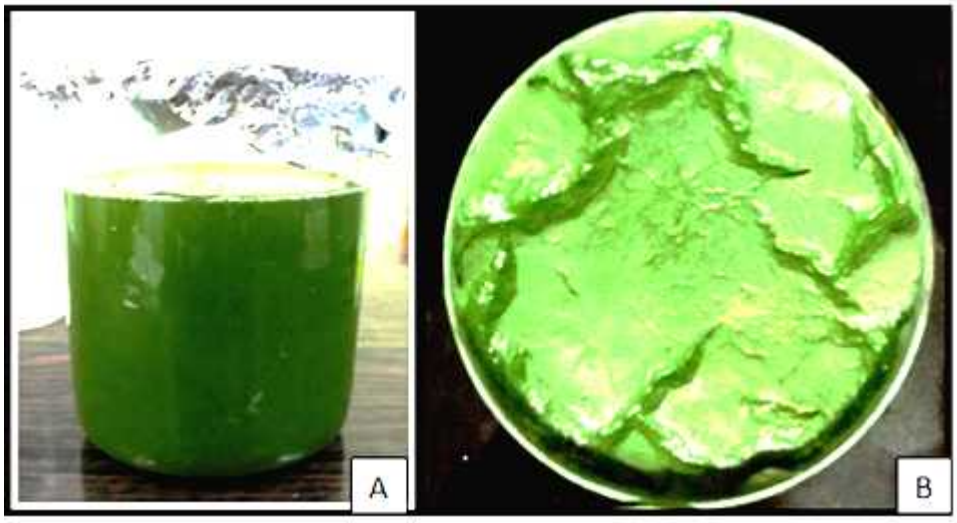

Figure: 3. *Microcystis aeruginosa*: A. Water collected from Sagar Lake. B. Filtered *Microcystis* cells.