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The Effects of Cyanobacterium *Microcystis aeruginosa* toxin on Nain (*Cirrhinus mrigala*)

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Abstract
The cyanotoxins are a diverse group of natural toxins, most of the cyanotoxins appear to be hazardous aquatic biota. In present study the crude cyanotoxin of Microcystis aeruginosa isolates was preliminarily characterized by estimating zone of inhibition of Pseudomonas solanacearum and Xanthomonas compestris on nutrient agar by sensitivity disc method. All the isolated inhibited the growth; however, maximum inhibition was recorded by isolate SPM 15. The concentration of microcystin in the different isolates of M. aeruginosa was evaluated by comparing the peaks with the standard. The result shows that isolate SPM 15 was highly toxic and contains maximum microcystin ca. 3200 µg g^{-1} dry weight followed by .SPM7. A significant (P=0.05) positive correlation was observed in the microcystin concentration and population of M. aeruginosa. For example, maximum growth was obtained at 30 ^{o}C (5.8 log cells ml^{-1}) corresponding to maximum microcystin value 3200 µg g^{-1} dry weight, the r^2 value was 0.91. The effect of acute, lethal and sub lethal concentrations of Microcystis were manifested by restlessness and equilibrium disturbance, rapid jerky body movements, erratic swimming, increased opercular movement and respiratory rate indicating difficulty in respiration as well as excess mucus secretion from general body surface of fish. These symptoms observed in the Nain were reversible after 24h exposure tovarious concentrations of toxin (microcystin). Safe Concentrations (presumably harmless) of toxin (microcystin) for the Nain was also evaluated.

Keywords: *Cyanotoxin, Cirrhinus mrigala, Microcystis, cyanobacteria, lake Surha*

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Introduction

Cyanobacteria are aquatic and photosynthetic organisms which live in the water, and can manufacture their own food. Many species of cyanobacteria possess gas vesicles. These are cytoplasmic inclusions that enable buoyancy regulation and are gas-filled, cylindrical structures. Their function is to give planktonic species an ecologically important mechanism enabling them to adjust their vertical position in the water column. Their extensive growth can create considerable nuisance for management of inland waters and they also release substances into the water which may be unpleasant (Jüttner, 1987) or toxic (cyanotoxins) (Gorham and Carmichael, 1988; Martins *et al.*, 2005). The cyanotoxins are a diverse group of natural toxins, both from the chemical and the toxicological points of view. In spite of their aquatic origin, most of the cyanotoxins that have been identified to date appear to be more hazardous to aquatic biota. Cyanobacteria produce a variety of unusual metabolites, the natural function of which is unclear, although some, perhaps only coincidentally, elicit effects upon other biota. Research has primarily focused on compounds that impact upon humans and livestock, either as toxins or as pharmaceutically useful substances. Globally the most frequently found cyanobacterial toxins in blooms from fresh and brackish waters are the cyclic peptide toxins of the microcystin and nodularin family. The toxicity of cyanobacterial mass occurrences (blooms) was originally brought to the attention of scientists through reports of animal poisonings by farmers and veterinarians. In most, if not all, reported cases since that time, afflicted animals consumed water from water bodies where there was an obvious presence of a cyanobacterial scum on the water surface (Watanabe*et al.* 1986). Others reported evidence suggests that immersion in toxic cyanobacteria or cyanotoxins may be harmful to fish. The mrigal (Nain; *Cirrhinus mrigala*) is the most widely farmed species among the Indian major carps of the Indo-Gangetic floodplains of Bangladesh, India and Pakistan which usually encounters the toxicity and eutrophication by cyanobacterial bloom (Jhingran and Khan. 1979). Therefore, present work focus on

collection of the cyanobacterial strains from different sites of the famous Suraha Lake used for commercial production of fishes, evaluation of presence of toxin in the samples, and study their effect on Nain *Cirrhinus mrigala*. Mainly the effect of crude extract of cyanobacteria on the different physiological behaviour of *Cirrhinus mrigala*was examined.

Materials and Methods

Toxin analysis of Microcystis aeruginosa

Isolates of *Microcystis aeruginosa*, isolated from Lake Surha of Ballia Utter Pradesh was grown on B-12 media. The *M. aeruginosa* biomass from exponential phase cultures was collected through centrifugation (1000 *g*) and stored at -20 ^{o}C. The crude extract containing toxin was prepared in aqueous and organic phase by re-suspending the harvested biomass (25 mg/ml) in distilled water for 30 minutes at room temperature. After incubation the samples were centrifuged at 3000 r.p.m. and aqueous extract was taken by pipetting. The organic extractions were made using methanol and ethyl acetatein acid instead of water. The crude toxin was subjected to bioassay.

Toxin Bioassay

I. Bacterial bioassays

In this study, bacterial bioassays with aqueous and organic extracts were performed as pour-plate methods. The test microorganisms: *Pseudomonas fluorescens* and *Xanthomonas compestris* were obtained from mycology lab of SMM Town PG College, Ballia. The plates were incubated at 30 °C for 24-60 h. Two to three colonies of bacteria were transferred to nutrient broth and incubated at 30 °C for 5 h. Colony count of the microorganisms was related to absorbance at 610 nm, and an appropriate amount of broth culture diluted in sterile phosphate-buffered saline (PBS) added to molten test agar. The bacterial count in the test agar was about 50 x 10^{-1} and 12-20 x 10^{-1} cfu ml^{-1}. Aliquots (8ml) of test agar were transferred to sterile Petri-dishes (100 mm dia). The discs containing water or organic extracts were placed over the agar plates and inhibition zones were measured and expressed as the distance in mm from the edge of filter disc to the point of normal colony size of the test microorganism.

II.. Quantifying antimicrobial effects, estimating minimum inhibitory concentration (MIC)

Duplicate tests with 10-fold and twofold dilutions of extracts from *M. aeruginosa* in methanol were carried out. The dose response relationship between the amount of freeze-dried cyanobacteria and the inhibition zones was measured. The calculated amount of freeze-dried cyanobacterial material present in the most diluted extract that produced a visible zone of inhibition was defined as the MIC. The stability of aqueous and methanol extracts was tested, aqueous extracts were kept at room temperature for 5 d. Filter discs with methanol extracts were stored in Petri dishes at room temperature for 5 weeks and the inhibition zones were measured at weekly intervals as described above.

Toxin Extraction and Testing

The toxin was extracted by the method described by Siegelman *et al.* (1984). In brief lyophilized cells were stirred in 20-200 ml of 5% n-butanol/20% methanol (v/v) in water for 1-2 h at 4 ^{o}C. The suspension was filtered through Whatman filter disc and alcohol content of filtrate was evaporated at 40 ^{o}C in rotary vacuum evaporator. The samples were eluted from C-18 bond-pack columns and elute was finally separated by HPLC (Waters) using a reverse phase column in acetonitrile-ammonium acetate solvent at the flow rate of 1.5 ml/min at 240 nm. The peaks published by Ohtake *et al.*, (1989), were used as reference standard.

Evaluation of effects on Cirrhinus mrigala

Studies were conducted using Nain (*Cirrhinus mrigala*) with mean weight 200 ±25 g. They were obtained from a fish pond of Agriculture farm of SMM Town PG College, Ballia and transferred to the laboratory where they were held in Fish tanks (8 individuals $tank^{-1}$) with 96 l of fresh water. Exposure to chlorine was minimized by filling the tanks at least 3 days before the fish were introduced. The temperature was kept constant (21 ±2 ^{o}C). The fishwere held in a 14 h: 10 h light:dark photoperiod. Mean values for additional parameters of water quality were: pH 7.6 ±0.2, Ca^{2+} 0.60 *mM* l^{-1} and Mg^{2+} 0.3 0.60 *mM* l^{-1}. Fish were fed with commercial fish food and were acclimatized for 7 days before the beginning of the experiments. Fish were not fed 24h prior transfer into experimental jars for acute static toxicity tests (Eisler, 1971). This was done because fecal matter and uneaten food could affect the biological activity of some toxic agents and increase biological oxygen demand (U.S. EPA, 1985). All possible care was taken to avoid giving stress to the fish during their transfer.

I. Acute Toxicity Studies

Acute toxicity tests with toxins from *M. aeruginosa* included the determination of median lethal concentrations at 24, 48, 72 and 96h under static test conditions. The tests were conducted basically as recommended by the committee on methods for toxicity tests with aquatic organisms (APHA *et al*., 1998). Each test involved the exposure of test organisms to a logarithmic series of six concentrations (dilution factor between concentrations about 50%). Ten sexually mature fish randomly selected and transferred to glass aquarium containing 80 l municipal tap water. The fish were exposed to toxin by feeding with *M. aeruginosa* cells under laboratory conditions for different periods of time to each of the concentrations which ranged from 25 mg to 800 mg (equivalent to 75 µg and 2400 µg toxin, respectively). The Fish were fed with commercial fish food plus freeze dried *M. aeruginosa* cells, containing toxin 3200 µg/g cells. *M. aeruginosa* cells were fed to the fish by manually crushing mixture of both components (fish food and toxic cells) in a mortar followed by sonication. This procedure resulted in small sticky pellets and was designed to replicate the type of exposure that may

occur when a bloom of cyanobacteria under-goes lysis under field conditions. The pellets were placed in the tank and drifted to the bottom for the fish to take. It was ensured that all the pellets were eaten within an hour. The amount of commercial fish food administered per fish was 0.3 g/day and the quantity of cyanobacterial cells was selected in order to dose approximately 75.00 μg to 2400 μg toxin per day. Six replicates of each concentrations were run to record the mortality data. The method of Litchfield and Wilcoxon (1949) was used to calculate the 24, 48, 72 and 96h LC_{50} values and 95% confidence limits. The LC_0 and LC_{100} values were also recorded for these time intervals by visual observations. The LC_0 values represented the highest concentrations tested which caused no death; LC_{100} values were the lowest concentrations which killed all the fish at a particular time interval. Assays were terminated and the results discarded if the control mortality exceeded 10%at any time. Fish were observed at two hour intervals during normal working hours for behavioral changes and survival.

Mortality of fish was recorded daily throughout the study. Dead fish during the bioassays were recorded and removed daily as they could deplete the dissolved oxygen of the media, thereby affecting the toxicity data (Schreck and Brouha, 1975). Death of the fish was confirmed when the movement of the operculum was stopped and there was no response from the fish when it was gently prodded. Parallel groups of control fish were held in municipal tap water.

II. Safe Concentrations

Presumably harmless (safe) concentrations of the toxin were estimated by the formula

$$C = (48\ h\ LC_{50} \times A)/S^2$$

Where C = presumably harmless (safe) concentration; A= Application factor; S= 48 h LC_{50}

The application factor (A) suggested by them was 0.3 which has been substituted in the above formula for calculating the concentration of toxin for *Cirrhinus mrighala.*

Statistical Analysis

All experiments were repeated at least twice. Water analysis, growth experiment, toxin analysis and acute toxicity studies consisted of 6 replicates and data were analyzed for standard deviation. In glass aquarium experiments each fish constituted an experimental unit and each unit was replicated 6 times within each treatment.

Results

The exponential phase biomass (from day 14 cultures) of *M. aeruginosa* SPM 15 obtained from the experiments; temperature, pH, light, nitrate and phosphate deficiency were freeze dried and used for microcystin estimation.

Table 1. Effect of *Microcystis aeruginosa* on growth inhibition of Microorganisms

Microorganisms	Zone of Inhibition (mm)							
	Aqueous extract				Methanol Extract			
	SPM 7	SPM9	SPM15	SPM 19	SPM 7	SPM9	SPM15	SPM19
Pseudomonas solanacearum	4	2	10	2	12	8	22	10
Xanthomonas compestris	2	1	5	1	6	5	10	6

Toxin Bioassay

The toxin in crude water or methanol extracts of *M. aeruginosa* isolates was preliminarily characterized by estimating zone of inhibition of *Pseudomonas solanacearum* and *Xanthomonas compestris* on nutrient agar by sensitivity disc method. All the isolated inhibited the growth; however, maximum inhibition was recorded by isolate SPM 15 (Table 1). In general, methanol extracts have more pronounced effect. For example, the zone of inhibition in *P. solanacearum* was 22 and 10 mm by *M. aeruginosa* SPM15 methanol and aqueous extracts, respectively. The minmum inhibitory concentration (MIC) for sensitivity control disc assay was also estimated. For *P. solanacearum* the MIC value was 66, 84, 32 and 72 mg ml^{-1} of *M. aeruginosa* isolates SPM7, SPM9, SPM15 and SPM19, respectively (Table 2).

Table 2: MIC value of *Microcystis aeruginosa* isolates for different microorganisms.

Microorganisms	Minimum inhibitory Concentration (mg/ml)			
	SPM 7	SPM9	SPM15	SPM19
Pseudomonas solanacearum	66	84	32	72
Xanthomonas compestris	73	94	45	80

The concentration of microcystin in the different isolates of *M. aeruginosa* was evaluated by comparing the peaks with the standard. The result shows that isolate SPM 15 was highly toxic and contains maximum microcystin ca. 3200 μg g^{-1} dry weight followed by .SPM7 (965 μg), SPM 19 (438 μg) and SPM 9 (257 μg; Table 3).

Table 3: Concentration of Microcystin in different strains of *Microcystis aeruginosa.*

Microcystis aeruginosa islates	Concentration (µg/g dry weight)
SPM7	965± 34.2
SPM9	257± 21.6
SPM15	3200± 65
SPM19	438± 51.7

The relationship between the amount of microcystin and biomass of *M. aeruginosa* cells in different growth conditions (Temperature, pH, nitrate and phosphate) was estimated (Fig. 1). In general, a significant (P=0.05) positive correlation was observed in the microcystin concentration and population of *M. aeruginosa.* For example, maximum growth was obtained at 30 °C (5.8 log cells ml^{-1}) corresponding to maximum microcystin value 3200 µg g^{-1} dry weight, the r^2 value was 0.91 (Fig. 1A). Similarly, the population was 6.5 with microcystin concentration 3150 µg g^{-1} dry weight at 70 µE m^{-2} s^{-1}, the r^2 value was 0.72 (Fig. 1B). The nitrate and phosphate also exhibited similar pattern of microcystin concentration (Fig. 1C, D). The r^2 values were 0.98 and 0.99 for nitrate and phosphate, respectively.

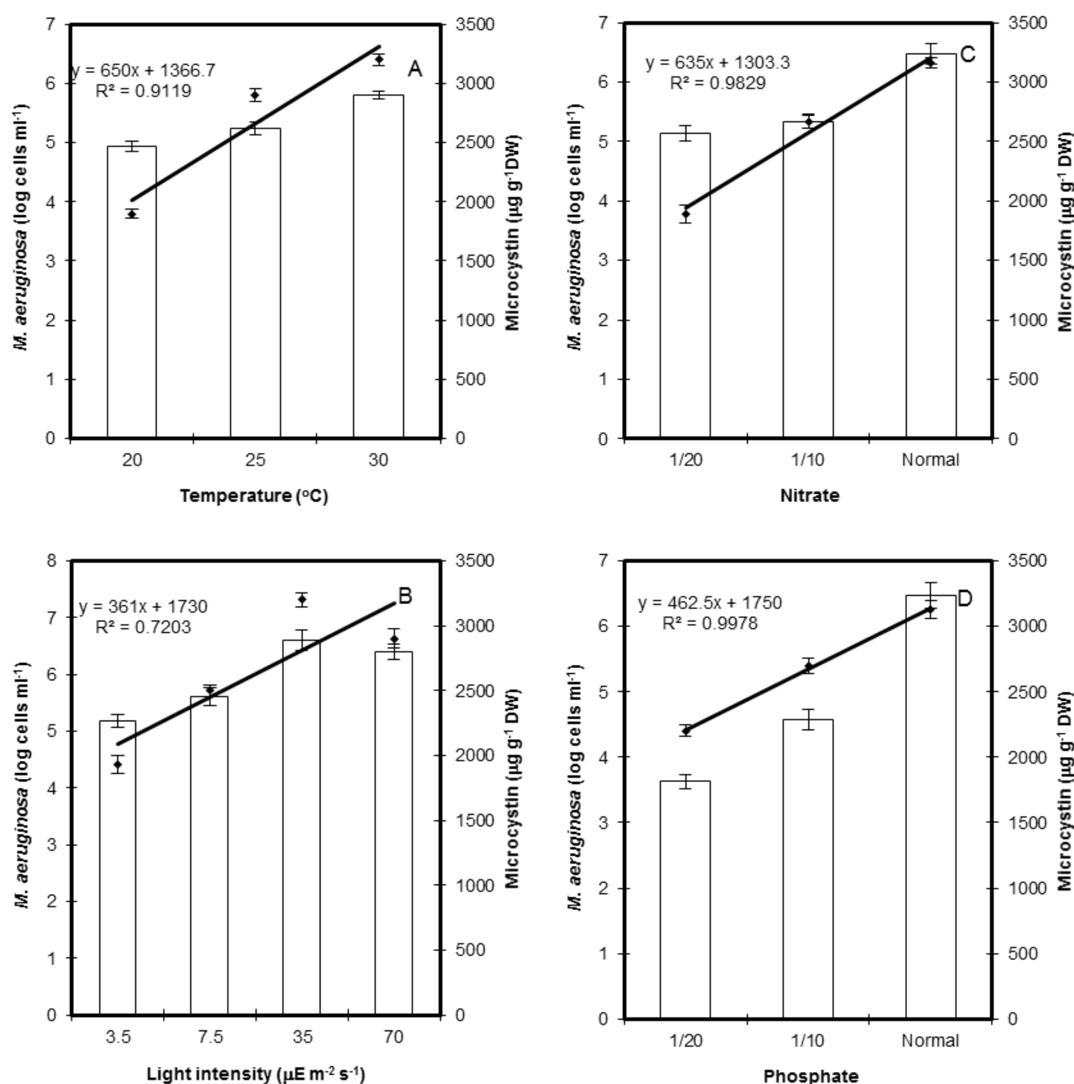

Fig. 1: Relationship between the environmental conditions and microcystin production by *M. aeruginosa* isolate SPM 9. A. Temperature; B. light intensity, C. Nitrate and D. Phosphate. Bars represent the number of cells (log) ml^{-1} in exponential phase cultures (10 d old) and symbols represent the amount of microcystin g^{-1} dry weight of cells of corresponding treatment. ; |= S.D.

Acute toxicity studies on cirrhinus mrigala

The effect of acute, lethal and sub lethal concentrations of *Microcystis* were manifested by restlessness and equilibrium disturbance, rapid jerky body movements, erratic swimming, increased opercular movement and respiratory rate indicating difficulty in respiration as well as excess mucus secretion from general body surface of fish. These symptoms observed in the Nain were reversible after 24h exposure to various concentrations of toxin (microcystin). Table 4 shows LC_0, LC_{50} and LC_{100} values (µg Kg^{-1} body weight) of

microcystin of *M. aeruginosa* SPM15 for the Nain (*Cirrhinus mrigala*)at four time invervals. The 24, 48, 72 and 96h. LC_{50} values for the Nain were found to be 2175, 670, 490, 450 µg. Kg^{-1} body weight, respectively. Whereas, the LC_0 values were 1100, 540, 400 and 310 µg. Kg^{-1} body weight, respectively. The LC_{100} values for similar time intervals were 2400, 850, 650, 525 µg. Kg^{-1} body weight, respectively (Table 4).

Table 4: The lethal concentrations of microcystin for Nain (*Cirrhinus mrigala*)

Observation time (h)	**Lethal Concentration**					
	LC_0		LC_{50}		LC_{100}	
	Microcystin µg Kg^{-1} BW	*SPM15 cells (mg)	Microcystin µg Kg^{-1} BW	*SPM15 cells (mg)	Microcystin µg Kg^{-1} BW	***SPM15 cells (mg)**
24	1100	375	2175 (1750-2450)	725	2400	**800**
48	540	180	670 (550-810)	223	850	**283**
72	400	133	490 (410-610)	163	650	**217**
96	**310**	**103**	**450 (340-535)**	**150**	**525**	**175**

** The amount of M. aeruginosa SPM15 cells (mg) equivalent to corresponding microcystin amount. The toxin was fed to the fish by mixing the cells with commercial feed. See material and methods for detail. 95% confidence limits are given in parenthesis. The data are mean of six replicates; each fish constituted an experimental unit.*

Safe Concentration

Safe Concentrations (presumably harmless) of toxin (microcystin) for the Nain was evaluated. $1/20^{th}$ of the LC_{50} value *i.e.* 22.50 µg. Kg^{-1} body weight was recorded as safe concentration (Table 12). The lethal and sublethal concentration of microcystin was estimated as $1/10^{th}$ and $1/15^{th}$ of LC50 value *i.e.* 45 and 30 µg. Kg^{-1}, respectively (Table 5).

Table 5: The acute, lethal, sub leathal, and safe concentrations of *M. aeruginosa* SPM15 to Nain (*Cirrhinus mrigala*).

LC_{50} value (µg)	**Safe Concentration**		**Lethal Concentration**		**Sub Lethal Concentration**	
	Concentration	*fraction	Concentration	*fraction	Concentration	***fraction**
450	**22.50**	**$1/20^{th}$**	**45**	**$1/10^{th}$**	**30**	**$1/15^{th}$**

** fraction of 96h LC_{50} value.*

Discussion

It was suggested that microcystin-producing strains always contain a minimum but do not exceed maximum cellular microcystin content (Long*et al.*, 2001) and that toxigenic strains remain so under a variety of growth conditions (Sivonen and Jones, 1999). Orr *et al.*, 1998;Sivonen and Jones, 1999 reported that highest peptide concentrations were produced under the medium or high light conditions and with the medium phosphate levels, conditions which promoted optimal or slightly suboptimal growth. The same has been true for microcystins released in water. In present study the behaviour of the Nain (*Cirrhinus mrigala*)observed was similar to those observed by various workers for teleostean species under influence of several water pollutants (Schoettger, 1970; Mishra and Srivastava, 1984; Srivastava *et al.*, 1995; Srivastava, *et al.* 1998b; Prashanth *et al.*, 2005). The avoidance reaction by the fish has been one of the more commonly mentioned parameters in behavioural studies with toxicants. The fish exhibited restlessnes, equilibrium disturbance, erratic swimming, increased opercular movement and excessive secretion of mucus from general body surface. It may, however, be pointed out that the toxicities of the individual toxicant to different species of fish are difficult to compare (Schimmel and Wilson, 1977) because they are influenced by various factors such as temperature pH, hardness and dissolved oxygen of test water (Schoettger, 1970; Smith and Sutton 1993; Gluth and Hanke 1983; Mushigeri and David , 2004). In the present study hypoproteinemia was observed in fish exposed to acute, lethal or sub lethal concentrations of microcystins containing cells of *M. aeruginosa*. Grant and Mehrle (1973), Folmar *et al.* (1993), Srivastava and Srivastava (1995a) and Srivastava *et al.* (1996a), found significant decrease in serum protein levels of fish exposed to chlorinated insecticides, pulp mill effluents and dyes. This study revealed that the microcystin contained in cyanobacterial blooms induce oxidative stress in Nain (*Cirrhinus mrigala*) that are exposed to repeated doses of toxins. The antioxidant status of the fish changed although no observable effects were detected in this species. Cyanobacteria can be a part of the diet of several species of fish (Bowen, 1982) and high numbers of toxic *Microcystis* cells have been recorded in fish (*Oreochromis niloticus* L.) guts (Mohamed *et al.*, 2003).

Conclusion

In conclusion the study revealed that environmental factors have a positive effect on microcystin production and content in *Microcystis* isolate SPM 15 up to the point where the maximum growth rate is reached. Histopathologic findings indicate that the liver is the primary site in the acute toxicity of this toxin. The results of this study showed that when Nain (*Cirrhinus mrigala*) were exposed to cyanobacterial cells under laboratory conditions exhibits induced toxicity

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