INTERNATIONAL JOURNAL OF ADVANCE RESEARCH, IDEAS AND INNOVATIONS IN TECHNOLOGY

ISSN: 2454-132X
Impact factor: 4.295
(Volume 4, Issue 4)
Available online at: www.ijariit.com

Effect of microsystin toxicity on Nain (Cirrhinus mrigala H.)

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ABSTRACT

Microcystis aeruginosa, cyanobacteria are frequent components of much fresh water marine ecosystem which often produce a hepatotoxin called microsytin. The toxin adversely affects the aquatic life forms, in the present study, its effect on Nain (Cirrhinus mrigala) was observed in biological parameters. In the fishes, the serum calcium after exposure to an acute concentration of microcystin for 96h, the values decreased significantly (P=0.05) to 9.9 mg 100 ml-1 compared to control (12.6 mg 100 ml-1). The acute exposure of microcystin also decreased the serum protein from 7.3 to 4.8. The fish showed hypercholesterolemia on exposure to acute and lethal concentrations of microcystin for 96h and both short (15-30 days) and long (45-90 days) term, respectively. The antioxidants, SOD activity did not change significantly in the liver, kidney or gills of fish that had been exposed to crushed cyanobacteria for 15 dthe ays at lethal concentration, however, the longer exposure (30 days) resulted in a significant increase (P=0.05) in the SOD activity in liver but induction in kidney and gills were not significant.

***Keywords**: Cirrhinus mrigala, Microcystein, Serum protein, Lipid peroxidase, SOD*

1. INTRODUCTION

The mrigal (*Cirrhinus mrigala* H.) is the most widely farmed species among the Indian major carps of the Indo-Gangetic floodplains of Bangladesh, India, and Pakistan. It is an important component of carp polyculture throughout South Asia. It was introduced for aquaculture, together with catla (*Catla catla*) and rohu (*Labeo rohita*), to other areas of India beyond its natural range in the early 1940's and in the 1950's and 1960's to other Asian countries (Jhingran 1982). Sixty-six reservoirs with an area of 137 034 ha are distributed among 17 districts in Uttar Pradesh. In addition, many small reservoirs have been documented with a total area of 20 845 ha, dispersed almost uniformly in the districts of U.P. Surha lake in district Ballia is one among them and is known for farming of Indian carps. Blue-green algae from the mainstay of plankton community in the vast majority of the man-made lakes studied. The overwhelming presence of *Microcystis aeruginosa* in Indian reservoirs is remarkable. The productive water of Gangetic plains invariably has a good standing crop of *Microcystis*. A common feature of all these reservoirs is the bright sunshine. *Microcystis* 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. Direct cyanobacterial poisoning of animals can occur by two routes: through consumption of cyanobacterial cells from the water, or indirectly through consumption of other animals that have themselves fed on cyanobacteria and accumulated cyanotoxins. Cyanotoxins are known to bioaccumulate in common aquatic vertebrates and invertebrates, including fish, mussels and zooplankton and plants (Beattie *et al.,* 1998; Mitrovic *et al*., 2005) Tencalla *et al.* (1994) showed that gastrointestinal uptake by gavage (force-feeding) caused massive hepatic necrosis followed by fish deaths, whereas immersion of adults and juveniles in contaminated water did not cause toxic effects. Others reported evidence suggests that immersion in toxic cyanobacteria or cyanotoxins may be harmful to fish. The release of toxic compounds from mass developments of cyanobacteria was considered to be the cause of fish kills by Penaloza *et al.* (1990). Histopathological investigations of fish deaths during cyanobacterial blooms in the UK indicated that the cause of death was mostly due to damage of the gills, digestive tract and liver (Rodger *et al.,* 1994; Cazenave *et al*., 2005). However, gill damage may have enhanced microcystin uptake and thus led to liver necrosis. Damage to gills by dissolved microcystin-LR has been shown experimentally in *Tilapia* and trout (Garcia, 1989; Gaete *et al.,* 1994; Bury *et al.,* 1996). Other pathological symptoms ascribed to toxic cyanobacterial blooms include damage to the liver, heart, kidney, gills, skin, and spleen (Garcia, 1989; Råbergh *et al.,* 1991). In the present work, the effect of microcystein from the *Microcystis aeruginosa* strains isolated from Surha lake of Ballia, U.P. on *Cirrhinus mrigala* was studied by exposing them *in vitro* to cyanobacterial strains for short and long terms and examined on various biochemical hematological parameters.

2. MATERIALS AND METHODS

2.1 Experimental Organism

The cyanobacterial and water samples were collected from 4 different sites of lake Surha, Ballia. The cyanobacterial species from the water were purified and cultured on B-12 media following dilution plating and single colony isolations (Nakagawa *et al.*,

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1987). Identified and purified isolates of *Microcystis aeruginosa* were maintained on the same medium. The fish, Nain (*Cirrhinus mrigala*) were caught from the fish pond of Agriculture farm of SMM Town PG College, Ballia and transferred to glass aquarium. The toxin from cultured *Microsystis* was extracted by the method described by Siegelman *et al.* (1984).

Acute toxicity and safe concentrations of microcystein for *C. mrigala* was determined 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 of about 50%) over eight set of fishes.

2.2 Test procedure for biochemical studies

Fish were subjected to acute (1/5th of 96h LC50), lethal ($1/10^{th}$ of 96 h LC_{50}) and sub lethal (1/15th of 96h LC_{50}) concentrations of toxins of *M. seruginosa.* A. group of 24 fish with holding densities 6 fish/10 L glass aqauarium (Srivastava and Sahai, 1987) was subjected to the following experimental design.

Group I : Control (0 days)
Group II : Acute concentration ($1/5^{th}$ of 96h LC_{50} value) for acute (96h) exposures.
Group III : Lethal concentrations ($1/10^{th}$ of 96h LC_{50} value) for short (15-30 days) and long (45-90 days) terms.
Group IV : Sub lethal concentrations ($1/15^{th}$ of 96h LC_{50} value) for short (15-30 days) and long (45-90 days) terms.

Six fish from each group including controls were sampled at specific time intervals. However, in the cases of both short and long term exposures, the fish were fed to satiation daily, but the food was discontinued 24h prior sampling of the fish. The water of the experimental tank was changed daily and pre-calculated amount of *M. aeruginosa* cell equivalent to corresponding toxin value was added daily to experimental tanks. After termination of an experiment of a particular time interval, fish were picked out from the aquaria and sampled for various biochemical and histological observations. Corresponding groups of control fish were also sampled at same time intervals

2.3 Sampling procedure

Fish were washed in clean water and anaesthetised (0.2 % phenoxyethanol) before the start of sampling. Fish were weighed, measured (total length) and blood was collected from the caudal vein into heparinized eppendorf tubes. Blood was centrifuged (4 min at 10 000 ***g*** at 4°C) and plasma was divided into aliquots and stored at -20°C until use. Fish were killed by decapitation, gills, liver, kidney, and gonads were carefully removed and weighed. A small piece of the liver and ovary was quick-frozen in liquid N_2 and stored at -20°C for histological analysis. Rest was used for biochemical analysis as given below:

Biochemical analysis

(i) Blood

Serum calcium and protein level were analyzed according to the procedure described by Trinder (1960) and Lowry *et al.* (1951), respectively. For measuring total blood cholesterol level blood was directly collected in citrated tuberculin syringes and analyzed by the method of Zlatkis *et al.* (1953).

(ii) Preparation of post-mitochondrial supernatant (PMS)

The liver, kidney, and gills were assayed for lipid peroxidase, catalase, and super oxide dismutase activity. The gill filaments of both sides were trimmed from the gill arches and the arches were discarded. The tissues (liver, kidney, and gill) were homogenized in chilled TRIS buffer (100 mM, pH 7.8; 1:10 w/v) using an electrically operated tissue homogenizer (Tanco). The homogenates were centrifuged at 10 500 ×*g* for 20 min at 4 ºC to obtain the post-mitochondrial supernatant (PMS) for various biochemical analyses.

a. Lipid peroxidation

The malondialdehyde (MDA) concentration, an index of lipid peroxidation, was measured. The homogenized tissue (0.5 ml), previously treated with 25 µl of butyl-hydroxytoluene 1% v/v in glacial acetic, was mixed with 0.2 ml of sodium laurylsulphate (8%), 1 ml of acetic acid (20% v/v) and 1 ml of 0.8% thiobarbituric acid. This mixture was then heated at 95 ºC for 30 min. The resulting chromogen was extracted with 3 ml of *n* -butyl alcohol and, after centrifugation (1500 ×*g* for 10 min), the absorbance of the organic phase was determined at 532 nm. 1, 1, 3, 3-Tetramethoxypropan was used as a standard. Values were presented as µmol MDA formed/g tissue.

b. Antioxidant enzymes

i. Catalase activity was assayed by the method of Beers and Sizer (1952). The reduction of hydrogen peroxide was followed spectrophotometrically at 240 nm, using 1.0 ml quartz cuvettes. Results are expressed in terms of nmol H_2O_2 consumed/min/mg protein.
ii. Superoxide dismutase activity was measured using the xanthine oxidase–cytochrome C method as described by McCord and Fridovich (1969). The reactions between xanthine and xanthine oxidase, and 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride (INT) led to superoxide radicals which reacted to form a red colored formazan. This was used to determine the SOD activity. In the presence of SOD in the medium, superoxide radicals were removed and the formation of formazan was therefore inhibited. SOD activity was measured spectrophotometrically at 505 nm and calculated as inhibition percent of formazan formation.

All experiments were repeated at least twice. In glass aquarium experiments each fish constituted an experimental unit and each unit was replicated 6 times within each treatment.

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3. RESULTS

3.1 Serum Calcium

The serum calcium values ranged from 12.6 to 15 mg 100 ml^{-1}. (Figs. 1-3) in control fish. After exposure to an acute concentration of microcystin (crushed cells of *M. aeruginosa*) for 96h, the values decreased significantly (*P*=0.05) to 9.9 mg 100 ml^{-1} compared to control (12.6 mg 100 ml^{-1}; Fig. 1A). The lethal concentration of microcystin also decreased the serum calcium levels when exposed for both short (15-30 days) and long (45-90 days) terms. For example the serum calcium level was 11.6 and 13.2 mg 100 ml^{-1} after 30 and 90 day exposure compared to corresponding control value 14.3 and 14.2 mg 100 ml^{-1}, respectively (Fig. 2A) The sublethal concentration only decreased the serum calcium level of the blood after short term but had no effect after long-term exposure (Fig. 3A).

3.2 Serum Protein

The average concentrations of serum protein values in control fish ranged between 7 to 8.2 g 100 ml^{-1} (Figs. 1-3). The acute exposure of microcystin decreased the serum protein from 7.3 to 4.8 (Fig. 1B). The lethal concentration also decreased the levels of serum protein during short and long-term exposure, however, the difference in level was more pronounced in short-term exposure. For example, the serum protein was 8 and 6.2 g 100 ml^{-1} after 30-day exposure to lethal concentration (Fig. 2B). The sub lethal concentrations of surfactants did not alter the level of serum protein significantly (*P* =0.05) at any time intervals (Fig. 3B).

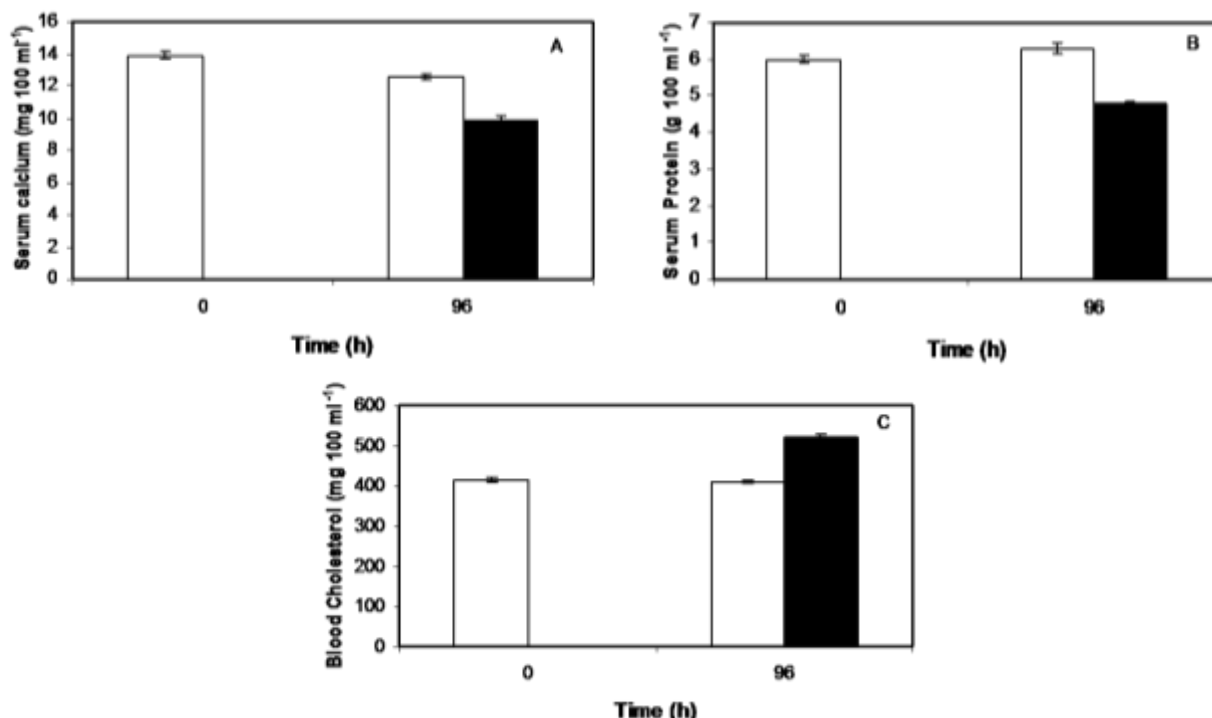
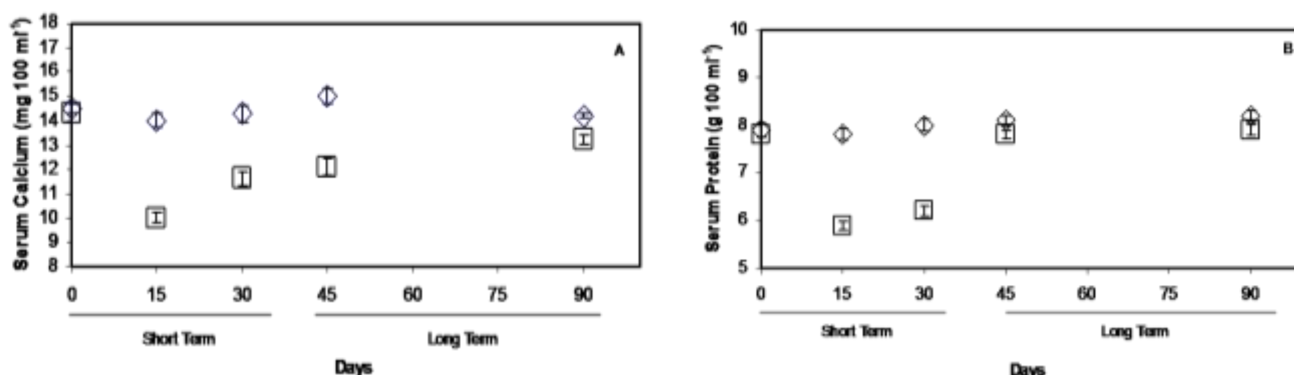


Fig. 1: Serum Calcium, Protein and Total Blood Cholesterol level of Nain (*Cirrhinus mrigala*) exposed to 96 h to acute concentration (1/5th of 96 h LC_{50} value) of *Microcystis aeruginosa*. A = Serum Calcium; B= Serum Protein; C = Blood Cholesterol. Bars represent □ Control and ■ treated. Data are mean of six replicates; | = S.D.

3.3 Total Blood Cholesterol

The total blood cholesterol levels in control fish *Cirrhinus mrigala* varies between 396 to 430 mg 100 ml^{-1} (Figs.1-3). The Nian showed hypercholesterolemia on exposure to acute and lethal concentrations of microcystin for 96h and both short (15-30 days) and long (45-90 days) term, respectively. (Figs. 1C and 3C). For example, the blood cholesterol was 410 and 523 mg 100 ml^{-1} in control and fish exposed to acute concentration for 96 h (Fig 1C). Similarly, the level was 396 and 500 mg 100 ml^{-1} after 15 days at lethal concentration. The sub lethal concentration also significantly (*P* =0.05) increased the level of total blood cholesterol for both short and long terms (Fig. 3C)



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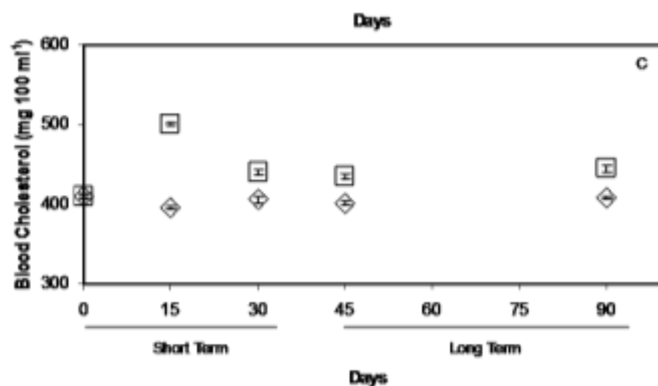

Fig. 2: Serum Calcium, Protein and Total Blood Cholesterol level of Nain (*Cirrhinus mrigala*) exposed for both short and long-term to lethal concentration (1/10th of 96 h LC_{50} value) of *Microcystis aeruginosa*. A = Serum Calcium; B= Serum Protein; C = Blood Cholesterol. Symbols represent ◊ Control and ☐ treated. Data are mean of six replicates; ǀ= S.D.

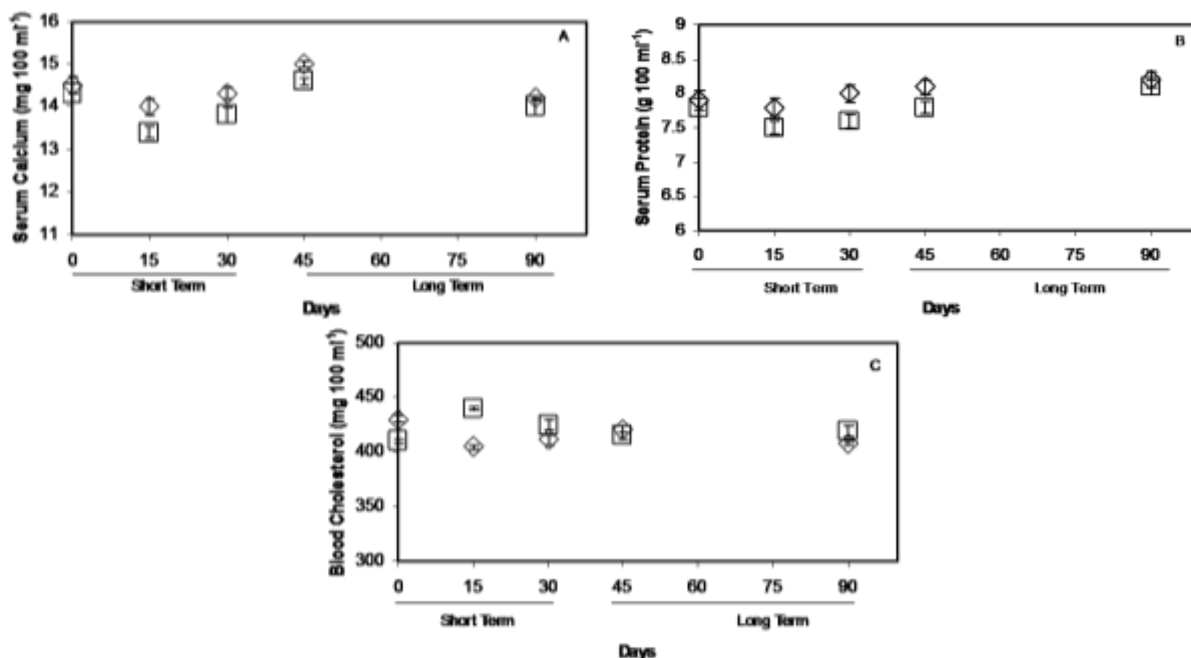

Fig. 3: Serum Calcium, Protein and Total Blood Cholesterol level of Nain (*Cirrhinus mrigala*) exposed for both short and long-term to sub lethal concentration (1/15th of 96 h LC_{50} value) of *Microcystis aeruginosa*. A = Serum Calcium; B= Serum Protein; C = Blood Cholesterol. Symbols represent ◊ Control and ☐ treated. Data are mean of six replicates; ǀ= S.D.

3.4 Lipid Peroxidation

After 15 days, no changes were observed in MDA in the fish exposed to lethal concentration when compared to the control group in any of the organs studied (Fig. 4). However, a longer exposure (30 days) in fish subjected to the crushed cyanobacteria significantly increased the value by 2.7, 3 and 1.3 folds in liver ($P = 0.01$), kidney ($P = 0.01$) and gills ($P = 0.05$; Fig. 4). The liver was the most affected organ.

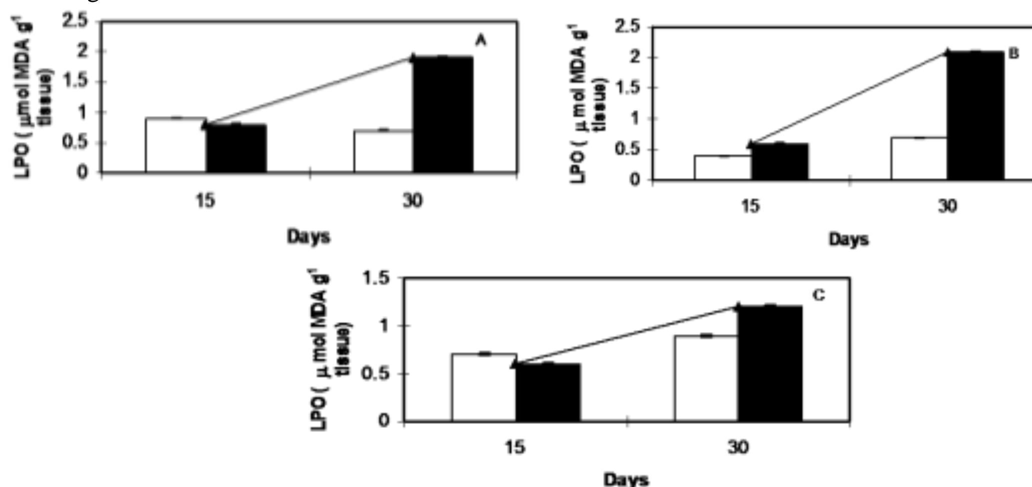

Fig. 4: Lipid peroxidation values in (a) liver, (b) kidney and (c) gills of control fish and fish exposed to lethal concentrations (1/10th of 96 h LC_{50} value) of *Microcystis aeruginosa* for short term. LPO values are expressed as µmol MDA g^{-1} tissue. Bars represent: ☐ Control and ■ treated. Data are mean of six replicates; ǀ= S.D.

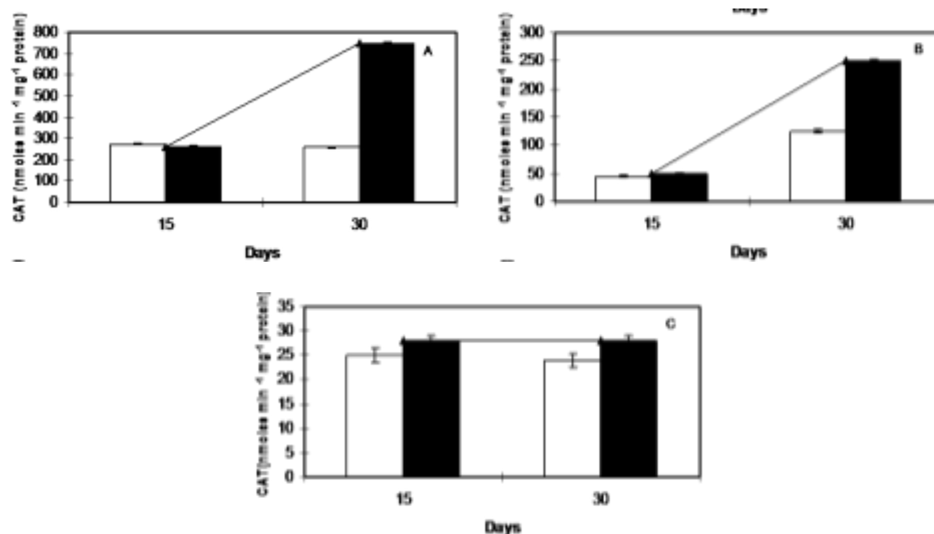

Fig. 5: Catalase activity in (a) liver, (b) kidney and (c) gills of control fish and fish exposed to lethal concentrations (1/10th of 96 h LC_{50} value) of *Microcystis aeruginosa* for short term. Catalase activity as expressed as nmole H_2O_2 consumed min^{-1} mg^{-1} protein. Bars represent: ☐ Control and ■ treated. Data are mean of six replicates; ⏐= S.D.

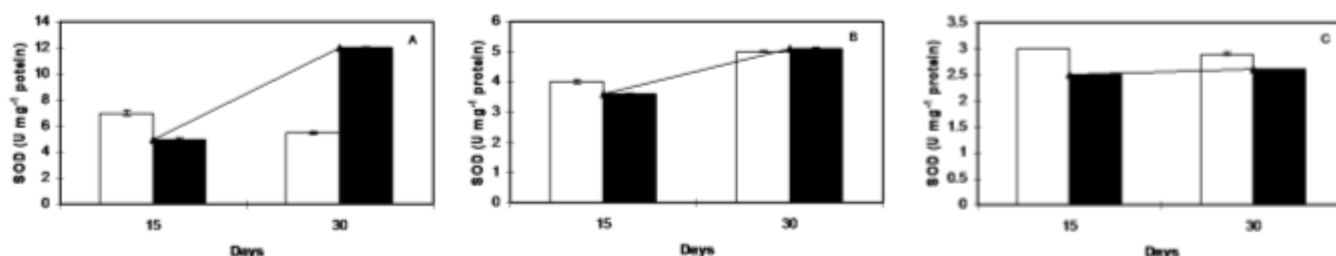

Fig.6: Super oxide dismutase activity in (a) liver, (b) kidney and (c) gills of control fish and fish exposed to lethal concentrations ((1/10th of 96 h LC_{50} value) of *Microcystis aeruginosa* for short term. SOD activity as expressed as U mg^{-1} protein. Bars represent: ☐ Control and ■ treated. Data are mean of six replicates; ⏐= S.D.

3.5. Antioxidant Enzymes

SOD activity did not change significantly in the liver, kidney or gills of fish that had been exposed to crushed cyanobacteria for 15 days at a lethal concentration (Fig. 5). The longer exposure (30 days) resulted in a significant increase (P=0.05) in the SOD activity in liver but induction in kidney and gills was not significant. For example, the SOD activity was 12 U mg^{-1} protein in liver compared to control (5 U mg^{-1} protein). Similarly, No discernible effects were observed in catalase activity of liver, kidney or gills after 15 days of exposure, but the activity increased 3 fold in liver (P= 0.01) and 2 fold in the kidney (P= 0.05) after 30 days of treatment (Fig. 6).

4. DISCUSSION

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. Microcystins have been characterised from planktonic *Anabaena, Microcystis, Oscillatoria (Planktothrix), Nostoc,* and *Anabaenopsis* species, and from terrestrial *Hapalosiphon* genera (Wiegand and Pflugmacher, 2005).

Microcystins After injection of sublethal doses of variously radiolabelled microcystins in mice and rats, about 70 per cent of the toxin was rapidly localised in the liver (Falconer *et al.,* 1986; Runnegar *et al.,* 1986a, b; Brooks and Codd, 1987; Robinson *et al.,* 1989, 1991; Meriluoto *et al.,* 1990;). In the present study, the change in serum calcium levels of fish on exposure to toxicants has been reported by several workers (Bansal *et al*., 1979; Verbost *et al*., 1987; Srivastava *et al*., 1995). Sharma *et al.* (1982) and Srivastava *et al.* (1995) reported an elevation of serum calcium level in *Heteropneustes fossilis* in response to dyes intoxication. In the present study also, serum calcium levels were found to decrease under the influence of microcystin. A possible cause of depeletion of calcium may be due to renal insufficiency and disrupted electrolyte balance. Hypoproteinemia was observed in fish exposed to acute, lethal or sub lethal concentrations of microcystins containing cells of *M. aeruginosa*. Hypercholesterolemia in the present study in fish exposed to microcystin may be due to the damage caused to the liver of the treated fish. A similar suggestion was made by Narain (1981) while providing guidelines for the interpretation of blood bioassays in fish. Hanke *et al.* (1983); Gluth and Hanke (1984, 1983) Srivastava and Narain (1985), Srivastava and Srivastava (1995 b) and Srivastava *et al.*, (1996a) reported an increase in total blood cholesterol level in fish on exposure to various insecticides at different time intervals. 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). Many studies have demonstrated that lipid peroxidation and oxidative stress increases in tissues of different species of aquatic organisms, as a result of being exposed to environmental stressors (Wiston and DiGiulio, 1991), but none were focused on microcystins. However, it is by no means a general rule that exposure to a pollutant increases the MDA level. As far as SOD and CAT activities are concerned, a simultaneous induction response is usually observed after exposure to pollutants (Dimitrova *et al.*, 1994).

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5. CONCLUSION

The results of this study showed that when Nain (*Cirrhinus mrigala*) were exposed to cyanobacterial cells under laboratory conditions the endogenous antioxidant defense system was altered in a time-dependent manner. The marked increase in blood cholesterol may be due to increased output of circulating levels of catecholamine during the stress of toxicosis. Simultaneously, the lipid peroxidation level increased significantly in the liver, kidney, and gills of the fish treated with microcystins containing crushed cyanobacterial cells. These findings suggest that oxidative stress plays an important role in *in vivo* microcystins induced toxicity in fish. More research is needed to elucidate the biochemistry of toxin production and its effect on aquatic animals.

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