EXCLI Journal 2006;5:66-78 – ISSN 1611-2156
Received: 16. June 2006, accepted: 24. June 2006, published 3. July 2006

Original article:

Hepatotoxicity of *Microcystis aeruginosa* Strains Growing as Blooms in Certain Eutrophic Ponds

Madhu B. Tyagi[1], Dhananjay P. Singh[2], Anil Kumar[2], Prabhat N. Jha[2], Rajeshwar P. Sinha[3], and Ashok Kumar[2*]

[1]Department of Botany, MMV, Banaras Hindu University, Varanasi- 221005, India; School of Biotechnology, Banaras Hindu University, Varanasi- 221005, India, Tel.: +91 542 2368331/2308334; Fax: +91 542 2368693 e-mail: kasokbt@rediffmail.com (*Corresponding author); [3]Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi- 221005, India

ABSTRACT

Critical assessment of five eutrophicated ponds of Varanasi city (India) revealed the presence of heavy blooms of cyanobacteria consisting mainly of *Microcystis aeruginosa*. Crude aqueous extracts of blooms as well as laboratory grown *M. aeruginosa* isolated from three ponds, namely Lakshmikund, Durgakund and Adityanagar showed toxicity in mouse bioassay test. Crude aqueous extracts from these samples caused death of test mice within 1h of administration (i.p.) with a LD_{50} of 60 mg/kg body weight and the treated animals showed clinical signs of hepatotoxicity. However such an effect was not associated with the blooms from Laatbhairov and Surajkund ponds suggesting that not all strains of *M. aeruginosa* are toxic. Based on spectral properties (λ_{max} 230 nm), and comparison with standard microcystin-LR, the toxin is tentatively identified as microcystin-LR. The purified toxin caused death of test mice within 40 min of its administration with a LD_{50} of 100 µg/ kg body weight and induced gross morphological and functional changes in liver. A 1.55 fold increase in liver weight accompanied by deep red coloration most probably due to hemorrhage and blood pooling suggested the hepatotoxic properties of the toxin. Hepatotoxicity was also evident from the drastic increase (up to 2.5 fold) in activity of serum enzymes such as glutamate pyruvate transaminase/alanine aminotransferase (GPT/ALT), lactate dehydrogenase (LDH) and alkaline phosphatase (APase) following toxin treatment. ^{14}C-labelling experiments demonstrated maximum accumulation (~15%) of ^{14}C- toxin after 20 min. of toxin administration. Appreciable level of toxin was also detected in water of four ponds. In conclusion these results clearly demonstrate that microcystin-producing blooms of *M. aeruginosa* are common in eutrophicated ponds of Varanasi city but not all ponds harbour toxic blooms.

Keywords: *Microcystis aeruginosa,* eutrophic ponds, hepatotoxicity, microcystin, serum enzymes

INTRODUCTION

Since the first report of toxic cyanobacteria (*Nodularia spumigena*) in the late 19th century (Francis, 1878) studies in several countries have revealed the wide occurrence of toxic cyanobacterial blooms causing serious problems in freshwater environment (Carmichael, 1992, 1994, Sivonen, 1996;

Codd et al. 1999; Tyagi et al., 1999; Nasri et al., 2004; Wiegand and Pflugmacher, 2005). The main toxic bloom-forming cyanobacterial strains belong to the genera *Anabaena, Microcystis, Nodularia, Nostoc* and *Oscillatoria* (*Planktothrix*). Two types of cyanobacterial toxins are known; (a) alkaloid neurotoxins (anatoxin-a, - a(s), saxitoxin and neosaxitoxin), which interfere with the functioning of the nervous system causing acute lethal toxicity due to paralysis of the respiratory muscles, and (b) cyclic peptide hepatotoxins (microcystin and nodularin), which damage the liver and result in excessive blood pooling in the liver, ultimately leading to fatal circulatory shock within a few hours or death within a few days (Theiss et al., 1988). Besides causing acute poisoning leading to death from massive hepatic hemorrhage, prolonged exposure to sublethal doses of microcystin induces primary hepatocellular carcinoma in rodents (Nishiwaki-Matsushima et al., 1992) and has been epidemiologically linked to primary liver cancer in humans (Yu, 1995). Of the known cyanotoxins, the hepatotoxic microcystins are the most widely distributed cyanotoxins and are produced by some strains of the genera *Microcystis, Anabaena, Oscillatoria* and *Nostoc*. However species of *Microcystis* are the most studied and toxic strains of this species have been reported from a number of countries. There are reports that different strains of *M. aeruginosa* species can be morphologically identical but differ in toxigenicity and may consist of both toxic and non-toxic strains (Meissner et al., 1996; Baker et al., 2002). Microcystin is synthesized non-ribosomally by multifunctional enzyme complex microcystin synthetase and recently the gene cluster encoding microcystin synthetase complex has been identified and sequenced (Baker et al., 2002). Characterization of 55 kb gene cluster of *mcy* genes (encoding microcystin synthetase) has led to the development of excellent molecular methods primarily based on PCR for screening of microcystin -producing strains of the genera *Anabaena, Microcystis* and *Planktothrix* (Hisbergues et al., 2003; Kurmayer et al., 2003).

In general all microcystins are monocyclic heptapeptides having the general structure cyclo (-D-Ala^{1}-X^{2}-D-Me Asp^{3}-Z^{4}-Adda^{5}-D-Glu^{6}-Mdha^{7}) in which X and Z are variable L-amino acids, D-MeAsp is D- erythro-β-methylaspartic acid, Mdha is N-methyldehydroalanine and Adda is a novel amino acid (2S, 3S, 8S, 9S)- 3-amino-9-methoxy-2, 6, 8-trimethyl-10 phenyldeca-4, 6-dienoic acid (Botes et al., 1983). Chemical variations such as different amino acid substitutions and a number of minor chemical modifications in the structure of microcystins are very common and over 60 variants are known; a single strain can produce more than one type of microcystin simultaneously (Rinehart et al., 1994; Codd et al., 1999; Mcelhiney and Lawton, 2005). These microcystins differ in their L-amino acid combinations and in having or lacking methyl group on amino acid 3, 5, and 7. Among all these variants microcystin–LR (leucine–arginine) has the widest geographical distribution (Codd et al., 1999; Tyagi et al., 1999). The molecular weight of microcystins varies from 909 to 1067 depending upon the variable L-amino acid present. Structural modifications to the Adda-glutamate region make microcystins non-toxic (Harada et al.,1988).

In spite of widespread occurrence of cyanobacterial blooms in eutrophic water bodies, no systematic work on the toxicity assessment of cyanobacterial blooms has been done. In our earlier study we observed that most temple ponds situated in the holy city of Varanasi (India) and nearby areas remain heavily infested with cyanobacterial blooms comprising of *M. aeruginosa* and/or other *Microcystis* spp. (Singh et al., 2001). However

the toxicity assessment and the impact of these toxic species on animals and human health have not been investigated. It is pertinent to mention that the water of these ponds is routinely and heavily used for bathing, drinking and other household needs and thus may pose serious health problems to humans and other animals. In view of recurrent appearance and/or persistence of blooms in these ponds and alarming health risks, we became interested to screen toxic species. The purpose of this investigation was to investigate: a) do all the ponds contain toxic cyanobacteria? b) are all the *M. aeruginosa* strains toxicogenic? and, c) is microcystin found in the water of various ponds ? Our results show that the ponds infested with heavy blooms of *M. aeruginosa* are indeed responsible for toxicity but all the strains even though morphologically identical are not toxic. We also report that different ponds do contain microcystin which may cause serious health problems.

MATERIALS AND METHODS

Organisms and growth conditions

Cyanobacterial blooms were collected from various eutrophic ponds situated in the central part of Varanasi. Based on morphological characteristics and available keys, the cyanobacterial genera present in blooms were identified (Desikachary, 1959). Unless otherwise stated, strains of *M. aeruginosa* species inhabiting different ponds were isolated and purified employing standard microbiological techniques. They were routinely grown in modified Jaworski medium (Thakur, 1996) at 25^{o} C in a culture room illuminated with Sylvania 40W T-12 cool white fluorescent light producing photosynthetically active radiation (PAR) of 14.4 W/m^2 from a distance of 50 cm. All other species associated with blooms were grown in unialgal form as described earlier (Thakur, 1996).

Preparation of crude aqueous extract

Natural unialgal bloom samples, consisting mostly of *M. aeruginosa* or laboratory grown *M. aeruginosa* isolated from different ponds were used for the preparation of crude aqueous extract. Lyophilized cells (1.0 g) were suspended in sterilized double distilled water and sonicated in a Branson sonifier 450 (Branson Ultrasonics Corp., Danbury, CT, USA) for 5 min at maximum out put and duty cycle. Sonicated suspension was centrifuged at 10,000 x g for 5 min; the resulting pellet was re-extracted if required. Supernatant was evaporated to dryness in vacuum and the dried material was dissolved in minimal volume of double distilled water to get desired concentrations of crude toxin.

Isolation, purification and estimation of toxin

Lyophilized cells (1.0 g) of *M. aeruginosa* were extracted thrice with 5 % aqueous acetic acid. Further separation and purification of toxin was done as described earlier (Harada et al., 1988) employing thin layer chromatography (TLC). Putative toxic spots were detected under short wave UV radiation (microcystins show absorption maxima at 238 nm). Desired spots were scratched from TLC plates and tested for toxicity to mice. Routine preparative TLC was employed for the enrichment of putative toxic spot which was then scratched from silica gel and recovered in methanol. Spectroscopic analysis of toxin present was made in an ATI Unicam UV/VIS spectrophotometer (Unicam Ltd., Cambridge, UK). The purity and identity of the toxic spot was checked by co-chromatography of the toxic fractions and the standard microcystin-LR (obtained as a gift from Prof. W.W. Carmichael, Wright State University, Dayton, OH, USA).

A high performance liquid chromatograph equipped with a constant flow pump (Millipore Water Lambda Max Model 481, Millipore Corp., USA) and LC spectrophotometer were used to purify the

toxin of *M. aeruginosa*. Separation was accomplished under reverse phase isocratic conditions with a Nucleosil-5C-18 column (4.6 x 250 mm) and methanol: trifluoroacetic acid (0.01 %) 61:39 (containing 0.8 % glycerol) as a mobile phase. Samples were run at a flow rate of 1.0 mL/ min and the separation was monitored with a variable wavelength UV detector operated at 238 nm. Presence of microcystin-LR, if any, in water of various ponds was determined using Quantiplate kit (Envrologix Inc., Portland, ME, USA) as per the instructions of the manufacturer.

Toxicity test

Known amounts of cyanobacterial extracts or purified toxins dissolved in normal saline (0.9 % NaCl) were injected intraperitoneally (i.p.) to male mice (Albino Park's strain weighing 20 ± 2 g) to evaluate the toxicity. Three mice per dose level and four different dose levels were selected. Survival times of less than 5 h were attributed to cyanobacterial toxicity. The median lethal dose (LD_{50}) value was taken as the concentration midway, between which, 100 % of test organisms survived and 100 % died.

Assay of serum enzyme activity

The activity of serum enzymes viz., alkaline phosphatase (APase), lactate dehydrogenase (LDH) and glutamate pyruvate transaminase/alanine amino transferase (GPT/ALT) was estimated following the methods as described earlier (Falconer et al., 1983) with certain modifications (Thakur, 1996). Estimations were made by enzyme kits obtained from Ranbaxy Laboratories Ltd., New Delhi. Serum was collected from mice treated with known doses of toxin. Test mice were food-starved for 6 h prior to the sample collection. Heparin was used as an anticoagulant.

Histopathological test

Tissues from liver of sacrificed mice (control and toxin administered) were washed with normal saline and were fixed in aqueous Bouin's fluid. The fixed tissues were dehydrated with the ascending concentrations of ethyl alcohol, cleared with xylene, and embedded in paraffin. The paraffin sections were cut at 6 µm and stained with Ehrlich haematoxylin/eosin.

^{14}C-labeling of toxin

In order to obtain ^{14}C-labeled toxin, 1.0 mL $NaH^{14}CO_3$ (specific activity 962 Bq mL^{-1} obtained from Radio-isotope group, Bhabha Atomic Research Centre, Trombay, Mumbai.) was added to 200 mL actively growing cultures of *M. aeruginosa* (OD = 0.4 at 663 nm). Cotton plug of culture vessel was replaced by stainless steel (SS) cap to avoid escape of $^{14}CO_2$. After 24 h, SS-caps were removed and 0.1 mL $NaH^{14}CO_3$ was added at 3- day intervals which lasted for 12 days. After 14 days cultures were harvested and toxin was extracted as described earlier. TLC of the crude extract was performed to confirm ^{14}C-incorporation in toxins. Spot showing toxicity was scratched from TLC plate and after processing counts present were determined in an LKB 1209 Rackbeta Liquid Scintillation Counter (LKB Wallac, Wallac OY, Turku, Finland).

Test for accumulation of ^{14}C-labeled toxin

^{14}C-labeled toxin, at a concentration of 100 µg/kg body weight, was injected to mice and ^{14}C incorporation in liver and spleen was monitored at desired time intervals after sacrificing the mice.

Statistical analysis

All the experiments were repeated at least three times with consistently the same conditions. Mean values and standard deviation were determined from three replicates for each experiment.

RESULTS

Screening of blooms for toxicity

Extensive survey and sampling of water from eutrophicated ponds of Varanasi were made to assess the formation and development of cyanobacterial blooms. Out of several ponds surveyed, five showed extensive development of cyanobacterial blooms (Table 1) either throughout the year or predominantly during summer. Among these ponds, bloom formation was most abundant in Lakshmikund followed by Durgakund. In fact Lakshmikund showed the presence of thick surface scum of blue-green colour almost throughout the year.

Table 1. Occurrence of cyanobacterial blooms in Varanasi[a] ponds.

Pond	Dominant bloom-forming cyanobacterium	Other cyanobacterial Genera	Toxicity[b]
Adityanagar	*Microcystis aeruginosa*	*Anabaenopsis, Nostoc, Plectonema*	+
Durgakund	*M. aeruginosa*	*Anabaenopsis, Oscillatoria, Plectonema, Raphidiopsis*	+
Laatbhairav	*M. aeruginosa*	*Nostoc, Oscillatoria*	–
Lakshmikund	*M. aeruginosa*	*Oscillatoria*	+
Surajkund	*M. aeruginosa*	*Anabaenopsis, Oscillatoria, Plectonema*	–

[a] Samples were collected during the years 2002 - 2003. At least 10 samples were collected randomly from each pond.

[b] 0.5 ml crude extract (approx. 1 mg protein/mL) of each bloom sample was used for toxicity assessment and response was recorded over 6 h. Results are based on three replicates.

Microscopic examination of algal blooms revealed that in all these ponds the blooms are mostly of the cyanobacterium *Microcystis* sp. The dominant bloom-forming species was tentatively identified as *M. aeruginosa* Kutz. All the strains of *M. aeruginosa* species from different ponds showed identical morphological features. Presence of other cyanobacterial genera such as *Anabaenopsis, Oscillatoria, Plectonema* and *Raphidiopsis* sp. was also noted although their abundance was invariably very low and they appeared only at some particular period of a season. In order to test the toxigenic property of natural bloom samples, crude aqueous extracts were subjected to mice bioassay test. Intraperitoneal injection (i.p.) of crude extracts of bloom samples collected from Adityanagar, Lakshmikund and Durgakund caused death of test animals within 1 h of toxin administration with a LD_{50} of 60 mg/kg body weight (Table 1). Similarly laboratory- grown *M. aeruginosa* strains originally isolated (separately) from the blooms of the above mentioned ponds also exhibited almost identical level of toxicity. The toxicity symptoms resembled closely with symptoms caused by known standard hepatotoxic *M. aeruginosa* strains. However unialgal cultures of other cyanobacteria present in blooms (Table 1) did not show any sign of toxicity in mice bioassay. On the other hand bloom samples from Surajkund and Laatbhairav did not show toxicity even after 6 h of toxin administration**.** Estimation of toxin level in water samples of above mentioned ponds showed a concentration of 1.15, 1.1, 0.74 and 0.22 µg /L of microcystin-LR in

Durgakund, Adityanagar Lakshmikund and Laatbhairov respectively.

Isolation and characterization of toxin

As routine sampling of selected ponds revealed that Lakshmikund is infested with blooms of *M. aeruginosa* almost throughout the year, all further experiments were conducted from the natural bloom samples or the strain of *M. aeruginosa* isolated from the bloom of this pond. Before conducting toxicological experiments, we attempted to isolate and purify the toxic fractions(s) present in the crude extract so as to determine the nature and degree of toxicity against the test organisms. Accordingly, a 5 % aqueous acetic acid extract of *M. aeruginosa* (bloom sample or laboratory- grown cultures) was subjected to thin layer chromatography on silica gel TLC plate. This resulted in the separation of three UV- fluorescent spots (MT-1 to MT-3) with Rf values of 0.54, 0.66 and 0.93 respectively (Table 2). When individually tested for toxicity, only the spot MT-1 proved to be toxic. Upon rechromatography of the pooled fraction of MT-1, a single spot with identical Rf value (0.54) reappeared on the TLC plate. The compound present in spot MT-1 showed absorption maximum at 230 nm (in methanol) and caused killing of mice within 40 min of toxin administration with a LD_{50} of 100 µg/kg body wt. (Table 2) thereby confirming its toxicogenic property. Furthermore toxin-treated mice showed clinical signs of poisoning such as restlessness, spasmodic leaping, fast breathing, slow movement, loss of co-ordination and splaying of hind limbs. Co-chromatography on TLC plates using standard microcystin-LR revealed a close mobility between the toxic constituent in MT-1 and standard microcystin-LR (Rf 0.54, absorption maximum 238 nm). The identity of the toxic constituent present in spot MT-1 was further confirmed by HPLC analysis (Fig. 1). It was observed that the retention time (t) of the standard microcystin-LR and toxic constituent of spot MT-1 were identical (11.4 min) under standard conditions of HPLC analyses. Based on chromatographic and spectral properties the compound isolated (present in spot MT-1) was tentatively identified as the microcystin (LR). Unless otherwise stated all further toxicity assessment studies were done with this purified compound.

Table 2. Separation of toxic fraction of *M. aeruginosa* by thin layer chromatography.

Spot No.	R*f*[a]	Recovery[b] (%)	Toxicity (LD_{50})[c]	λ_{max} (nm)	Identification
MT-1	0.54	0.21	100	230	Microcystin-LR[d]
MT-2	0.66	0.15	Nil	204	Unidentified
MT-3	0.93	0.12	Nil	220	Unidentified

Mobile phase- chloroform : methanol : water (65 : 35 : 10 v/v, lower layer).
[a] Detection under low wavelength UV radiation.
[b] Recovery based on dry wt. of algal cell.
[c] LD_{50} value is based on µg/kg body weight.
[d] Identification is based on comparison with standard purified microcystin-LR.

Toxin effect on liver weight and morphology

As expected, toxin administration into the test mice (100 µg/kg body weight) markedly increased the liver weight (1.55 fold) after 40 min of toxin administration. A deep red colour was acquired by the liver most probably due to hemorrhage and blood pooling. In addition autopsies showed a marked congestion in the liver of the sacrificed mice.

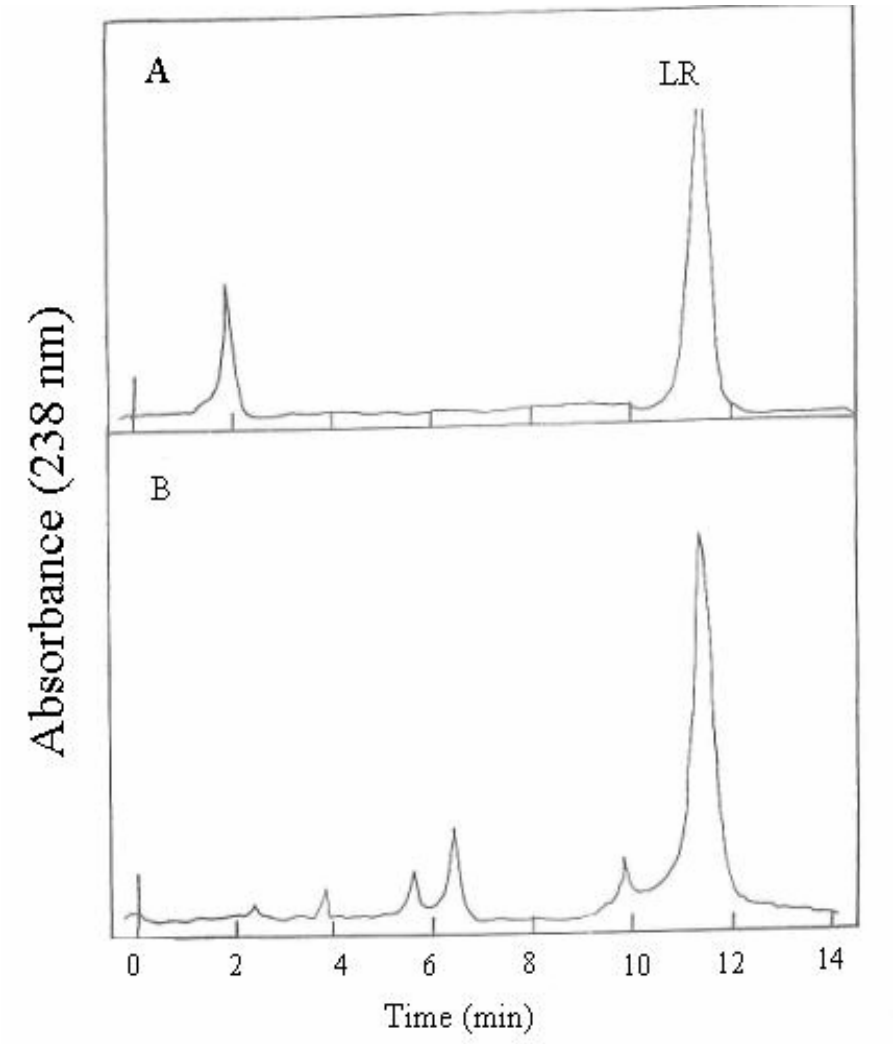


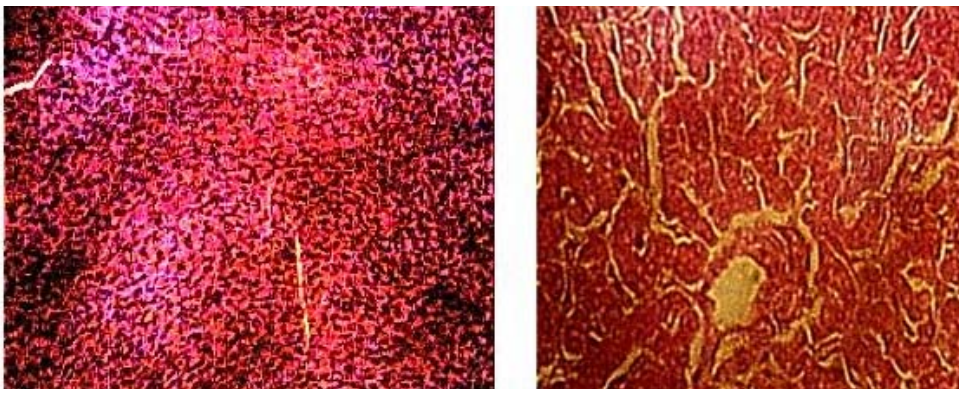
Figure 1. HPLC profile of standard microcystin-LR (A) and purified toxin of *M. aeruginosa* (B).

For details see Materials and Methods section

Histopathological examination of the liver revealed a swollen liver and centrilobular to panlobular hemorrhagic necrosis (Fig. 2). Altogether the liver suffered gross changes in its normal architecture.

Effect of toxin on serum enzyme activity

The hepatotoxic effect of the toxin was further confirmed by its effect on activity of three serum enzymes namely alanine amino transferase, lactate dehydrogenase, and alkaline phosphatase. A time course study after toxin administration revealed that the toxin caused a marked and gradual increase in the activity of the serum enzymes ALT, LDH and APase (Fig. 3). The maximum increase in the enzyme activity was observed after 40 min of the toxin administration which corresponded to 2.48, 2.50 and 2.27 fold increases respectively for ALT, LDH and APase. Administration of pond water as such did not cause significant increase in the level of above enzyme activity; however, if the toxin was concentrated in pond water, the activity increased.



A **B**

Figure 2. Photomicrographs of liver sections from control (A) and toxin- treated mice (B).

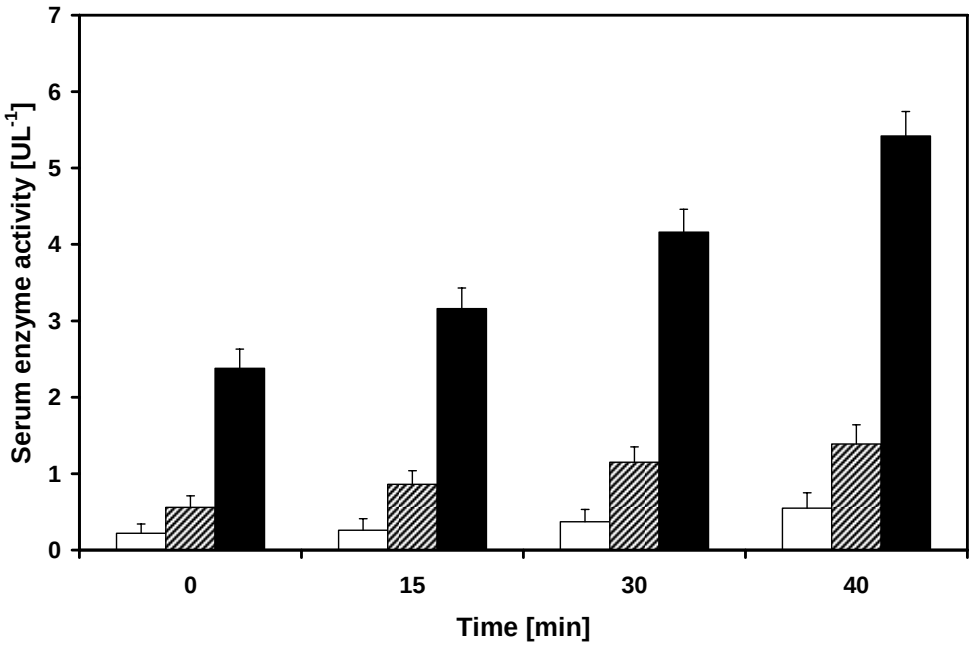


Figure 3: Effect of purified toxin on activity of mice serum enzymes. Lactate dehydrogenase (open bar), alanine aminotransferase (hatched bar), and alkaline phosphatase (black bar). The serum enzyme activity was measured at indicated time intervals after injecting 100 µg/kg body wt. of purified toxin. The basal enzyme activity of the control (untreated) mice, denoted at 0 time, remained more or less unchanged up to 40 min. Values are means of three separate but identical experiments ± SD.

Accumulation of labeled toxin in different organs

Once it became evident that the toxin administration induces gross structural, histopathological and functional changes in the liver, we attempted to study the accumulation of toxin in liver and spleen of mice. For this, the uptake of ^{14}C- labeled toxin in liver and

spleen was studied at desired time intervals after injecting toxin at a dose of 100 µg/kg body weight. The data of Fig. 4 shows that the accumulation of ^{14}C- labeled toxin occurred instantly and there was maximum uptake within 20 min in the liver whereafter the increase in counts was negligible. On the other hand the accumulation of toxin in the spleen was 6-7 fold lower in comparison to liver at 20 min; nevertheless uptake continued till 40 min. The data show that maximum incorporation occurred in liver (~15%) followed by spleen (5 - 6 %) after 40 min of toxin administration.

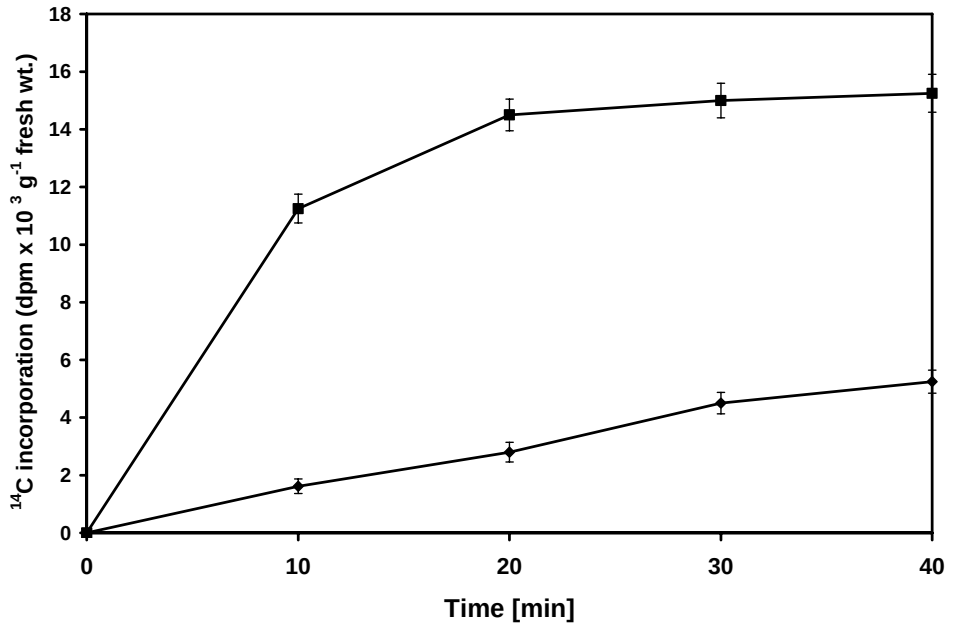


Figure 4: Incorporation of ^{14}C-labeled toxin into the liver (■) and spleen (♦) of mice. ^{14}C-labeled purified toxin (100 µg/kg body wt.) was injected into the mice and the incorporation of toxin into liver and spleen was determined at desired time intervals after sacrificing the mice. Total initial count in 0.5 ml toxin was 1 x 10^5 counts per min (CPM). Each value represents mean of three separate but identical experiments ± SD.

DISCUSSION

Toxic cyanobacterial blooms are found in many eutrophic to hypereutrophic lakes, ponds and rivers throughout the world and are responsible for the worldwide killing of wild and domestic animals, fishes and birds (Carmichael, 1992; Sivonen, 1996; Tyagi et al.,. 1999; Rao et al., 2002). Results so far obtained clearly reveal that hepatotoxic (microcystin-containing) blooms of cyanobacteria are more common than neurotoxic blooms, at least in fresh water environment. In the present investigation studies conducted in five eutrophic ponds of Varanasi have revealed the occurrence of thick cyanobacterial blooms comprising mainly of *Microcystis aeruginosa.* In general all these ponds are heavily infested with blooms of *M. aeruginosa* especially in summer months, excepting Lakshmikund in which *M. aeruginosa* blooms occur almost throughout the year. Changes in water depth and nutritional load may be responsible in regulating the abundance and appearance of blooms in different ponds (Carmichael, 1994; Thakur, 1996; Tyagi et al., 1999).

Mouse bioassay test for toxicity determination has revealed the toxigenic property of crude aqueous extracts of *M. aeruginosa* bloom samples from three ponds namely, Adityanagar, Lakshmikund and Durgakund. Treated mice died within 1 h of administration of crude extract (LD_{50} 60 mg/kg body weight) and upon necropsy the animals showed grossly enlarged liver engorged with blood. These preliminary results suggested the hepatotoxic nature of *M. aeruginosa* growing in above three ponds. However bloom samples from Laatbhairav and Surajkund ponds did not show any sign of toxicity. This is an interesting observation in view of the fact that blooms of these two ponds were also having dominance of *M. aeruginosa* and the strains were morphologically identical to those present in the other ponds. From these above results it emerges that not all strains of *M. aeruginosa* may always be associated with toxicity. Data of microcystin level present in water samples of various ponds also corroborate the incidence of toxicity. Concentration of microcystin-LR in Durgakund and Adityanagar was recorded at 1.15 and 1.10 µg/L, which is not safe as per the guidelines of World health organization Drinking water containing microcystin above 1 µg/L is forbidden for use**.** Presence of varying level of microcystin in different ponds might be due to the population size/abundance of *M. aeruginosa* in an individual pond.

Based on clinical symptoms and chromatographic/spectral properties, the toxin produced by *M. aeruginosa* strains reported herein appears to be the hepatotoxin microcystin. This assumption is based on the fact that the UV fluorescent spot MT-1 (Rf 0.54) seen by TLC was toxic to mice and its chromatographic pattern appeared identical to the standard microcystin-LR. Furthermore analysis of this compound by HPLC also revealed homology with standard microcystin-LR. In general microcystins show an absorption maximum at 238 nm (Harada et al. 1988). In our estimation a slight shift (λ_{max} 230 nm) was observed which might be due to some minor structural changes in the compound or some alteration occurred during the process of isolation. It is pertinent to mention that several structural variants of microcystins have been reported (Reinehart et al., 1994; Codd et al., 1999). Hepatotoxicity is also evident from the fact that the purified toxin from *M. aeruginosa* strains when bioassayed against test mice caused acute toxicity (LD_{50} 100 µg/kg body wt.; i.p.) with death of mice occurring at 40 min and the toxicity symptoms resembled those of standard microcystin-LR (Berg et al., 1987; Carmichel, 1994). Almost similar lethal dose values for microcystin in mice were reported by Harada et al., (1988) and Carmichel (1994).

The hepatotoxic effects of the isolated toxin are also supported by the morphological and histopathological effects on liver. Necropsy of toxin-administered mice showed grossly enlarged deep red coloured liver with extensive centrilobular to panlobular necrosis. Our results agree with those of Ito et. al. (1997) who reported liver necrosis in aged mice after oral administration of microcystin LR. Several workers have proposed that the ultimate cause of death by *M. aeruginosa* toxin may be primarily due to the hypovolemic shock brought about by massive intrahepatic hemorrhage (Falconer et al., 1983; Theiss et al., 1988; Pouria et al., 1998). The observation pertaining to an increase in liver weight (up to 1.55 fold ca. 55%) is in accordance with previous reports (Falconer et al., 1983) and points to the presence of microcystin in this *M. aeruginosa* strain.

That the effect of toxin is mediated directly through liver is evident from our observations showing maximum accumulation of the toxin into the liver. Runnegar et al. (1991) also suggested that the specificity of the microcystin for the liver may be due to the

selective transport of the toxin into the hepatocytes by multispecific bile acid transport system. Based on clinical property, clinical symptoms and data of toxin transport, it is concluded that the toxin is hepatotoxic.

Results showing an increase in serum enzyme activities after toxin administration also point to hepatocellular damage and dysfunction. In human beings also, an increase in alkaline phosphatase (APase) activity is generally observed in diseases of the liver and biliary tract, post-hepatic jaundice as well as infective or toxic hepatitis (Solter et al., 1998). Lactate dehydrogenase which is widely distributed in all organs/cells of the body shows a high activity in the liver and skeletal muscles. An increase in LDH activity is also observed in liver diseases (Carmichael, 1994) and is a useful indicator of microcystin LR hepatotoxicity (Falconer et al., 1983). Several workers have reported elevation of alanine aminotransferase in plasma specimens from people whose source of drinking water was a reservoir infested with heavy bloom of toxic *M. aeruginosa* (Falconer, et al., 1983; Carmichael, 1992, 1994).

In conclusion our results show that *M. aeruginosa* strains growing as a bloom in many eutrophic ponds are potent toxic cyanobacteria and solely produce microcystin. Microcystin is hepatotoxic in nature and death of test animals occurs mainly due to malfunctioning of liver. It is also evident that toxicity is not prevalent in all the strains of *M. aeruginosa* species in spite of having identical morphological characters. Furthermore, there is release of significant level of microcystin in pond water although concentration varies in various ponds. Due to its hepatotoxicity and relatively long half life, toxin from *M. aeruginosa* in water bodies pose a high risk for humans.

Acknowledgements: This work was partly supported by a research grant sanctioned to AK by the Department of Biotechnology, Government of India, New Delhi (BT/PR1239/AGR/02/065/98). We are thankful to Prof. W. W. Carmichael, USA, for sending us standard microcystin. Anil Kumar is thankful to UGC for the award of Junior Research Fellowship. PNJ is thankful to CSIR for the award of Senior Research Fellowship. We are grateful to Prof. H.D. Kumar, Ex. Professor of Botany, Banaras Hindu University, for his suggestions and critically reviewing the manuscript.

REFERENCES

Baker JA, Barrie EB, Neilan A, Mckay DB. Monitoring changing toxigenicity of a cyanobacterial bloom by molecular methods. *Appl Environ Microbiol* 2002; 68:6070-6

Berg K, Skulberg OM, Skulberg R. Effects of toxic blue-green algae on water quality – a laboratory study. *Arch Hydrobiol* 1987;108:549-63

Botes DP, Viljoen CC, Kruger H, Wessels PL, Williams DH. Structure of toxins of the blue-green alga *Microcystis aeruginosa. S Afr J Sci* 1982;78**:**378- 9

Carmichael WW. Cyanobacterial secondary metabolites- the cyanotoxins. *J Appl Bacteriol* 1992; 72:460-6

Carmichael WW. The toxins of cyanobacteria. *Sci Am* 1994;270:64-70

Codd GA, Bell SG, Kaya K, Ward CJ, Beattie KA, Metcalf JS. Cyanobacterial toxins and human health. *Eur J Phycol.* 1999; 34:405-15

Desikachary TV. *Cyanophyta.* Indian Council of Agricultural Research, New Delhi. 1959

Falconer IR. Tumor promotion and liver injury caused by oral consumption of cyanobacteria. *Environ Toxicol Water Quality* 1991;6:177-84

Falconer IR, Beresford AM, Runnegar MTC. Evidence of liver damage by toxin from a bloom of the blue-green alga *Microcystis aeruginosa*. *Med J Aust*, 1983;1:511-4

Francis, G. Poisonous Australian lake. *Nature* 1878;18:1-12

Harada K.-I, Suzuki M, Dahlem AM, Beasley VR, Carmichael WW, Rinehart KL. Improved method for purification of toxic peptides produced by cyanobacteria. *Toxicon* 1988;26:433- 9

Hisbergues M, Christiansen G, Rouhiainen L, Sivonen, K, Borner T. PCR-based identification of microcystin-producing genotypes of different cyanobacterial genera. *Arch Microbiol* 2003;180: 402-10

Ito E, Kondo F, Harada K-I. Hepatic necrosis in aged mice by oral administration of microcystin-LR. *Toxicon* 1997;35:231-9

Kurmayer R, Christiansen G, Chorus I. The abundance of microcystin producing genotypes correlated positively with colony size in *Microcystis* sp. and determines its microcystin net production in Lake Wannsee. *Appl Environ Microbiol* 2003;69:787-95

McElhiney J, Lawton LA. Detection of the cyanobacterial hepatotoxins microcystins. *Toxicol Appl Pharmacol* 2005;203:219-30

Meissner K, Dittmann E, Borner T. Toxic and non-toxic strains of the cyanobacterium *Microcystis aeruginosa* contain sequences homologous to peptide synthetase genes. *FEMS Microbiol Lett* 1996;135:295-303

Nasri AB, Bouaicha N, Fastner, J. First report of microcystin containing bloom of the cyanobacterium *Microcystis* spp. in Lake Qubeira, Eastern Algeria. *Arch Environ Contam Toxicol*, 2004;46:197-202

Nishiwaki-Matsushima, R, Ohto T, Nishiwaki S, Suganuma M, Kohyama K, Ishikawa T, Carmichael WW, Fujiki H. Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. *J Cancer Res Clin Oncol*, 1992;118:420- 4

Pouria S, Andrade A, Barbosa J, Cavalcanti RL, Barreto, VTS, Ward CJ, Preisner W, Poon GK, Neild GH, Codd GA. Fatal microcystin intoxication in haemodialysis unit in Caruaru (Brazil). *Lancet* 1998;352:21-6

Rao PV, Gupta N, Bhaskar AS, Jayaraj R. Toxins and bioactive compounds from cyanobacteria and their implications on human health. *J Environ Biol* 2002;23:215-24

Rinehart KL, Namikoshi M, Choi BW. Structure and biosynthesis of toxins from blue-green algae (cyanobacteria). *J Appl Phycol* 1994;6:159-76

Runnegar MTC, Gerdes RG, Falconer IR. The uptake of the cyanobacterial hepatotoxin microcystin by isolated rat hepatocytes. *Toxicon* 1991;29:43-51

Singh DP, Tyagi MB, Kumar A, Thakur JK, Kumar A. Antialgal activity of a hepatotoxin producing cyanobacterium, *Microcystis aeruginosa*. *World J Microbiol Biotechnol* 2001;17:15-22

Sivonen K. Cyanobacterial toxins and toxin production. *Phycologia* 1996;35:12-24

Solter PF, Wollenberg GK, Huang X, Chu FS, Runnegar MT. Prolonged sublethal exposure to the protein phosphatase inhibitor microcystin-LR results in multiple dose-dependent hepatotoxic effects. *Toxicol Sci* 1998;44:87- 96

Thakur JK. Physiological and biochemical studies on toxin production by cyanobacteria. Ph. D. Thesis, Banaras Hindu University, Varanasi, India. 1996

Theiss, WC., Carmichael WW, Wyman J, Bruner R. Blood pressure and hepatocellular effects of the cyclic heptapeptide toxin produced by *Microcystis aeruginosa* strain PCC-7820. *Toxicon* 1988:26:603- 13

Tyagi MB, Thakur JK, Singh DP, Kumar A, Prasuna EG, Kumar A. Cyanobacterial toxins: the

current status. *J Microbiol Biotechnol* 1999;9: 9-21

Wiegand W, Pflugmacher S. Ecotoxicological effects of selected cyanobacterial secondary metabolites: a short review. *Toxicol Appl Pharmacol* 2005;203:201-18

Yu S–Z. Primary prevention of hepatocellular carcinoma. *J Gastroenterol Hepatol* 1995;10: 674-82