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Occurrence of Microcystin-Containing Toxic Water Blooms in Central India

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Abstract Three out of fourteen *Microcystis*-dominant cyanobacterial blooms in Central India were found to be toxic to mice (LD_{50} ranging from 35–450 mg bloom dry mass/kg body weight). The liver architecture of the treated mice showed characteristic symptoms of hepatotoxicity relative to the untreated controls, with increased enzyme activities of serum lactate dehydrogenase (LDH), serum glutamate oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP), and serum glutamate pyruvate transaminase (SGPT). RP-HPLC revealed the presence of microcystin-LR, microcystin-RR, and desmethyl microcystin-RR in the given region to maximum amounts of 390, 1,030, and 860 μg/g bloom dry weight, respectively, corresponding to a maximum of 2.8 mg/l microcystin-LR in the lake water. Further confirmation of the microcystin variants was conducted using a MALDI-TOF MS analysis.

Key words: Cyanobacteria, hepatotoxicity, India, MALDI-TOF, microcystin variants, *Microcystis*-dominant blooms

The health hazards and economic losses associated with toxic cyanobacterial blooms are already well documented, including in South-East Asia [4, 5, 14, 28, 32]. The best-studied toxins, microcystins (MCYSTs), include over sixty different chemical variants of cyclic heptapeptides [28], produced by species of *Microcystis*, *Oscillatoria* (*Planktothrix*), *Nostoc*, *Anabaena*, *Hapalosiphon*, *Anabaenopsis*, and *Radiocystis* [28, 31]. MCYSTs are primarily hepatotoxins and liver-tumor-promoting agents, due to their inhibition of the regulatory enzymes protein phosphatases 1 and 2A [15, 18]. MCYST exposure is also toxic to the intestines [3], lungs [22], kidneys [2], and immune system [27], and leads to the formation of reactive oxygen species, resulting in cytoskeleton disruption [7] and the induction of apoptotic and DNA breakdown activities in a variety of cell cultures [9, 24].

Cyanobacterial toxins in drinking or recreational waters are also hazardous to human health. The high incidence of primary liver cancer in China [29] and the deaths of patients in a hemodialysis unit in Brazil [12] because of the consumption of MCYST-contaminated water are some examples of cyanobacterial poisoning. In India, the warm water temperature promotes dense *Microcystis* growth almost throughout the year [1, 21]. Although there is no evidence of human or animal lethality associated with these blooms, the occurrence of toxic blooms has been described in a few cases [16, 25]. In an epidemiologic study, it was found that patients with a history of bathing in *Microcystis*-infested ponds developed acute rhinosporidiosis, a disease caused by "pathogenic" strains of *Microcystis* [6]. Several *Microcystis*-dominant phytoplankton materials collected from Central India have also been found to be toxic to the crustacean zooplankton *Moina macrocopa* [1]. However, the possible cause of such toxicity, whether due to MCYSTs or some other toxins, has not yet been determined.

Accordingly, the present paper reports on the occurrence of MCYSTs in cyanobacterial blooms collected from water bodies in Central India, a region where people routinely consume untreated water from such sources.

Materials and Methods

Bloom Material

Bloom samples from ponds and lakes in Central India (Districts of Jabalpur, Dindori, Mandla, Seoni, and Shahdol; 79°E-81°E longitude and 22°N-24°N latitude) were collected by skimming across the water surface using a 25-μm plankton net in the early summer of 2001–2003. The samples were kept cool (10°C) and brought to the laboratory.

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The *Microcystis* spp. were microscopically identified [33], and their relative abundance determined using a hemocytometer according to Watanabe *et al.* [33]. Using different sieves, the large particles and zooplankton were separated. The phytoplankton samples were concentrated by placing the material in plastic cylinders and the buoyant cyanobacterial scum collected from the surface. The samples were then lyophilized and kept at −20°C until extraction.

Extraction of Crude MCYST Samples

The freeze-dried bloom material (10 g dry weight) was extracted with three batches of 250 ml of methanol. The supernatant was then evaporated *in vacuo,* and the residue dissolved in 10% methanol was applied to a preconditioned LiChrolut RP-18 (ODS) cartridge (500 mg sorbent, Merck, Germany). Thereafter, the sorbent was washed with 20% methanol and the bound material eluted with 90% methanol. This elute was then evaporated *in vacuo* and the residue re-dissolved in 1 ml of methanol.

Reverse-Phase HPLC Separation

The RP-HPLC was carried out according to Lawton et al. [13]. Briefly, the ODS eluates were diluted to a 50% methanol concentration and 50 µl of the sample injected into the RP-HPLC. The detection of MCYSTs was performed with a Nucleodur® CC250/4, 5 µm, C-18 gravity column (Macherey-Nagel, Germany) using a Waters 600 E multi-solvent delivery system. The mobile phase was a gradient of water, acetonitrile, and 0.05% trifluoroacetic acid in acetonitrile. The flow rate was 1 ml/min.

For the UV detection, a Waters 996 photodiode array detector (PDA) and Waters 600E controller (Waters, Germany) were used. The UV spectra were recorded between 200–300 nm, then the MCYSTs were detected according to their characteristic UV spectrum. The amount of MCYSTs was estimated according to Wirsing *et al.* [36] using a calibration curve of the MCYST-LR (the L-leucine, L-arginine variant) (5–1,000 ng, Calbiochem, Germany).

MALDI-TOF Mass Spectrometric Measurements

The procedure of Erhard *et al.* [8] was adopted. The freeze-dried bloom material (50 µg) was dissolved in 10 µl water/ethanol/acetonitrile (1:1:1).The samples were then analyzed in a saturated α-cyano-4-hydroxycinnamic acid matrix solubilized in 50% acetonitrile and 0.3% TFA. A mixture of 1 ml of the matrix and 1 ml of the sample was prepared directly on the plate. The samples were then analyzed using a MALDI VOYAGER DEPRO time-of-flight mass spectrometer (Perspective Biosystems, U.S.A.) equipped with a nitrogen laser (337 nm output). The ions were accelerated with a voltage of 20 kV and the measurements performed in a delayed extraction mode, allowing the determination of the mono-isotopic mass value. A low-mass gate of 400 improved the measurement by filtering out the most intensive matrix ions. The mass spectrometer was operated in the positive-ion detection and reflector mode.

Toxicity Bioassay and Biochemical Estimations

The bioassay for toxicity was conducted using the method of Ohtake *et al.* [19]. Two hundred mg (dry wt)/ml of a suspension of the freeze-dried bloom (in 0.9% saline) was sonicated (15 µ amplitude for 10 min, MSE-MK sonifier), centrifuged (10,000 ×*g*), and then membrane filtered (0.45 µm, Sartorius). The filtrate was used to determine a 50% lethal dose (LD_{50}) according to Weil [34], then quadruplicate mice (4-wk-old male albinos, weighing 28–30 g) were intraperitoneally injected successively with decreasing concentrations of the extract or equivalent saline (controls) until the lowest lethal concentration (*ca.*= LD_{100}) was attained. The extract was considered nontoxic when the mice survived 10 h or the dose was over 800 mg/kg body weight.

To test the biochemical parameters, extracts equivalent to *ca.* LD_{100} were injected in four mice. Blood was then collected in heparinized tubes from the retroorbital plexus 120 min after the treatment. The serum glutamate oxaloacetate transaminase (SGOT), glutamate pyruvate transaminase (SGPT), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) activities were all measured using a spectrophotometer (Beckman DU 640, U.S.A.) with commercial diagnostic kits (E. Merck India Ltd., India). The surviving mice were then sacrificed by cervical dislocation 4 h after the administration. Liver tissue samples were washed with normal saline and fixed in 10% buffered formalin. After proper fixation, the tissue was processed by dehydration and embedded in paraffin. Sections were then prepared and stained with hematoxylin and eosine for examination under a light microscope.

Triplicate analyses were performed with a control and four animals were treated in each batch (n=12). Unless otherwise stated, the average values and ±SE's are presented. Statistically significant differences between the treatments were determined using Student's *t*-test.

RESULTS AND DISCUSSION

Incidence of Cyanobacterial Blooms

A survey of eighty-odd permanent water bodies (in 1999) situated in Central India revealed the dominance of fourteen cyanobacterial water blooms comprised of different species of *Microcystis*. Bloom samples from three lakes situated in the towns of Kundam, Gosalpur, and Shahpura were found to be toxic to the zooplankter *Moina macrocopa* at concentrations >282 µg bloom dry wt/ml [1]. In 2001 and 2003, profuse blooms predominantly formed by *M. aeruginosa* and *M. viridis* were also noted at the same

Table 1. Relative abundance of three species of *Microcystis* and toxicity in bloom samples collected from different locations in Central India.

Location	Date of sampling	Hydrological characteristics	Relative abundance of *Microcystis* species (%)			50% lethal dose (mg dry wt. bloom/kg animal)
			-aeruginosa	*-viridis*	*-wesenbergii*	
Kundam	21.4.2001	38/10/292[a]	65	30	5	108±12
	13.3.2003		70	25	5	35±7
Gosalpur	25.4.2001	6.8/5/30	75	25	nd[b]	195±41
	19.3.2003		64	36	nd	48±12
Shahpura	29.4.2001	8.2/5/37	80	20	nd	450±32
	21.3.2003		75	25	nd	128±20

[a]Surface area (km^2)/mean depth (m)/water volume (10^6 m^3).
[b]nd, not detectable.

locations (>80% of total cells). The minor forms were *M. wesenbergii*, *Anabaena spiroides*, *Oscillatoria* sp., and *Phormidium* sp.

Bloom Toxicity

As shown in Table 1, out of the fourteen blooms studied, the occurrence of toxic blooms over two years was only detected at three locations; i.e., Kundam (23°13'N; 80°21'E, District Jabalpur), Gosalpur (23°30'N; 80°09'E, District Jabalpur), and Shahpura (23°11'N; 80°42'E, District Dindori). The hydrological features of the sites are given in Table 1 and the trophic levels included in Ref. [1]. The LD_{50} in 2003 at the different locations varied between 35–130 mg dry wt/kg. The mice usually died within 1.5–5 h of receiving the intraperitoneal dose preceded by marked sedation and a splaying of the hind limbs. The liver was the most affected among the organs. Upon necropsy, the liver was grossly distended and engorged with blood. The

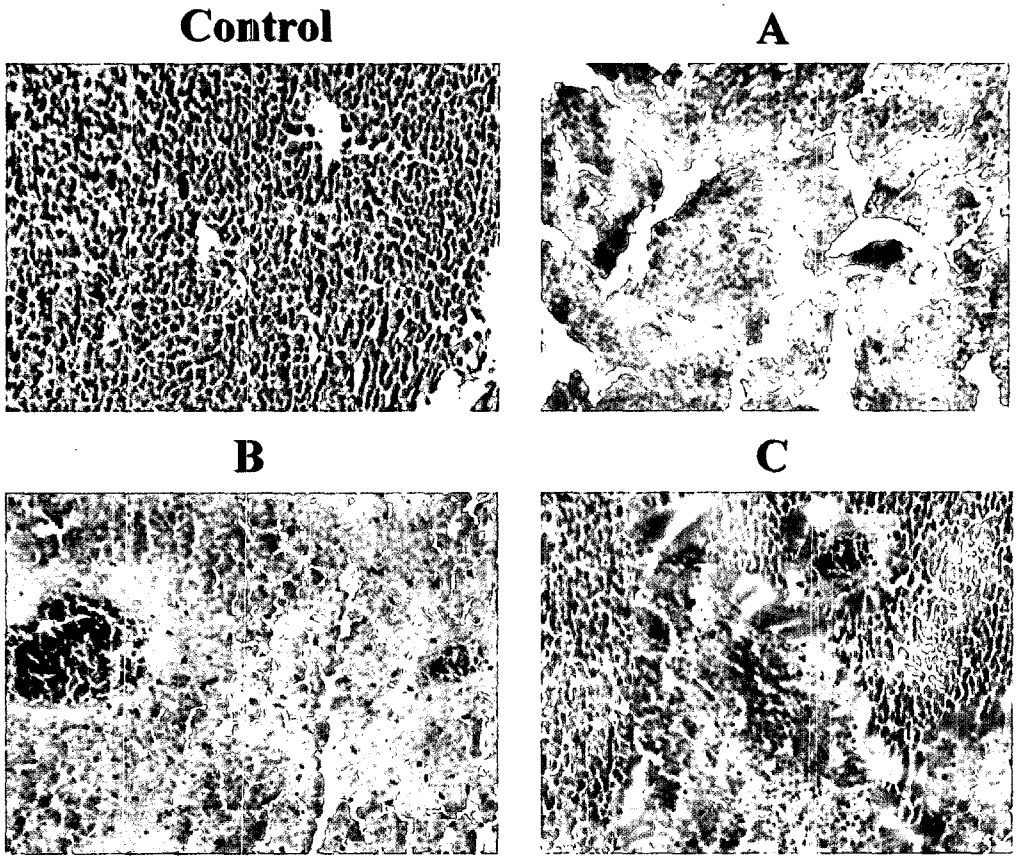

Fig. 1. Histomicrographs of perfused livers of mice injected with saline (control) or bloom extracts from Kundam (A), Gosalpur (B), or Shahpura (C) lake equivalent to *ca.* LD_{100} dose, as shown in text.

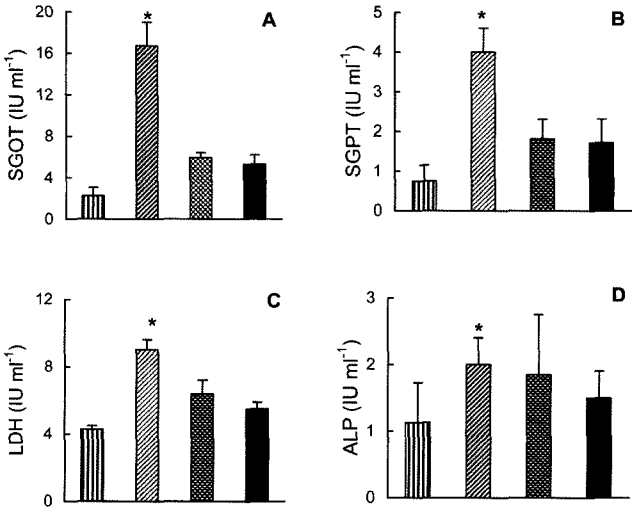

Fig. 2. Serum levels of hepatic enzymes, serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP), after i.p. administration of saline (▥), or *ca.* LD_{100} dose of bloom extracts from Kundam (▨), Gosalpur (▩), or Shahpura (■) lake.
*Significant from other treatments at $p<0.05$.

liver histology showed signs of hepatotoxicity characteristic of MCYSTs, such as degeneration and vacuolation of the hepatic parenchyma, congestion and hemorrhaging, and hepatic vacuolation, etc.

On the basis of an acute toxic dose (*ca.* LD_{100}), that is, 70, 100, and 260 mg dry wt/kg of the Kundam, Gosalpur, and Shahpura bloom material, respectively, exposure to the Kundam lake bloom caused the most severe pathological lesions in the hepatic tissue (Fig. 1). The effect of these bloom doses on the serum enzymes of hepatic origin, namely, SGOT, SGPT, LDH, and ALP, is shown in Fig. 2. At 120 min post-treatment in the case of just the Kundam lake bloom, a significant change ($p<0.05$) was observed in the enzymes relative to the control groups, suggesting excessive enzyme leakage. Meanwhile, under the same conditions, the administration of nontoxic bloom material equivalent to 1,000 mg dry wt/kg animal had no apparent effect on the hepatic architecture or activity levels of the serum enzymes (data not shown).

Isolation of MCYSTs by RP-HPLC

The RP-HPLC analysis of the three samples revealed several major peaks (Kundam 3.8, 5.7, 6.1, and 14.8 min; Gosalpur 5.7 and 21.7 min, and Shahpura 4.5, 4.6, 4.8, and 5.7 min), and all three samples exhibited a UV_{max} at 220 nm and 275 nm, characteristic of peptides in the PDA analysis (Fig. 3). Peaks at 5.7, 6.1, and 14.8 min typical of MCYSTs were detected and identified as MCYST-RR (the L-arginine, L-arginine variant); desmethyl MCYST-RR, and MCYST-LR based on their UV_{max} at 238.1, 242.9,

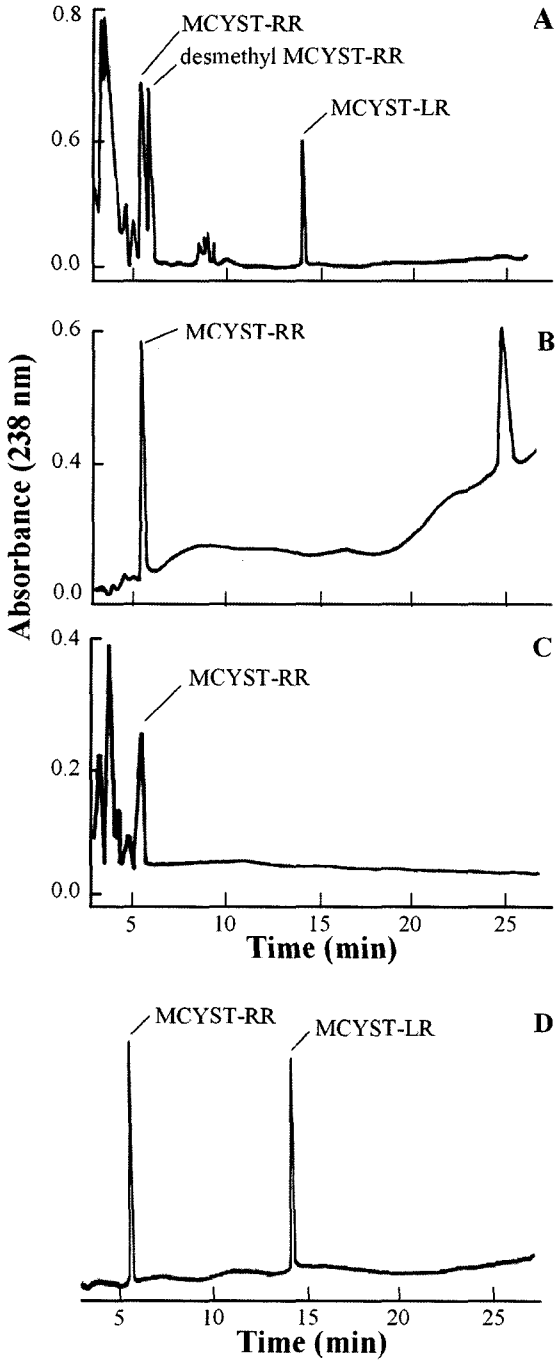

Fig. 3. RP-HPLC elution profiles of 2 mg bloom samples from Kundam (A), Gosalpur (B), and Shahpura (C) lake in 2003 after methanolic and solid-phase extraction and (D) standard MCYST-LR and -RR (20 ng each) in 50 µl methanol.

and 232.0 nm, respectively. The peak for MCYST-RR and -LR corresponded to the standards. The amounts of MCYST-RR, -desmethyl RR, and -LR in the Kundam bloom sample were 1,030, 860, and 390 µg/g dry weight, whereas the MCYST-RR in the other two bloom samples was within a range of 280–1,500 µg/g. The amount of

MCYSTs in the remaining blooms was below the HPLC detection limit.

Identification of MCYSTs by MALDI-TOF MS

When applying the MALDI-TOF MS analysis to whole cells, the three prominent ions, *m/z*=1,038.57, *m/z*=1,024.65, and *m/z*=995.68, observed in the bloom samples from Kundam lake (Fig. 4) were characteristic of MCYST-RR, desmethyl MCYST-RR, and MCYST-LR, respectively. In accordance with the HPLC results, MCYST-RR was also found in the samples from the other two lakes. The analysis of the Gosalpur lake samples further revealed the presence of linear peptides, presumably aeruginosins, although an exact assignment of the HPLC peaks was impossible owing to the lack of proper separation and unavailability of standards. As such, the trypsin inhibitory activity of bloom extracts previously reported by the current authors [1] was presumably due to the presence of such peptides.

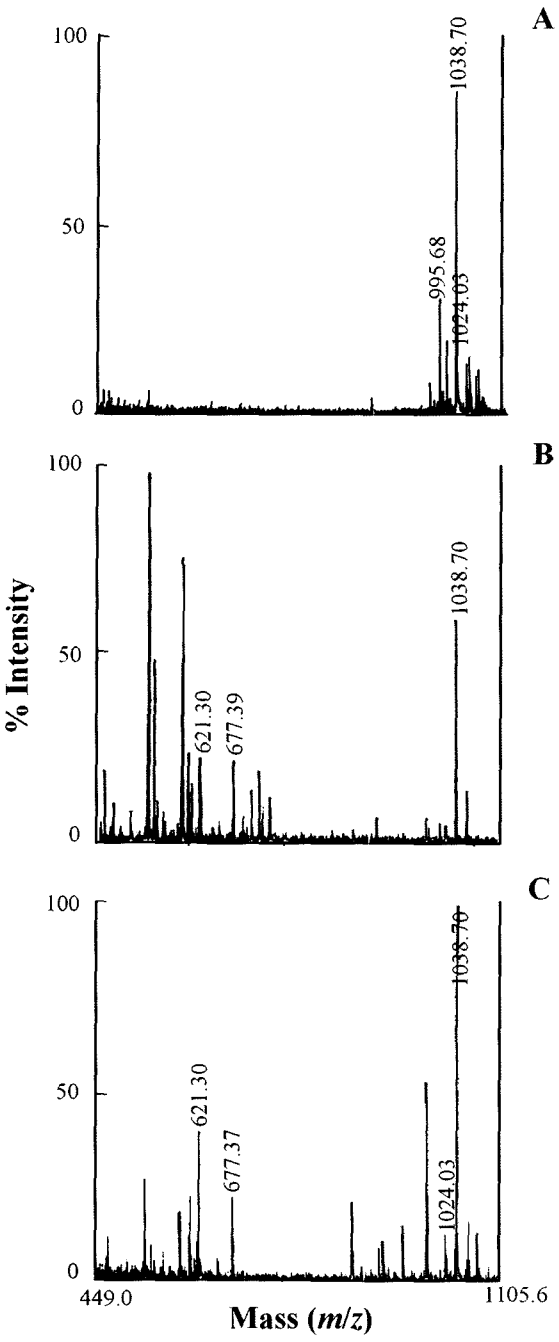

Fig. 4. MALDI-TOF MS of bloom samples from Kundam (A), Gosalpur (B), and Shahpura (C) lake in 2003. *m/z* 621.29 and 677.36=aeruginosins; *m/z* 995.68=MCYST-LR; *m/z* 1024.55= desmethyl MCYST-RR; and *m/z*=1038.57=MCYST-RR. The remaining fragments were not further analyzed.

Consequently, the present results reinforce earlier preliminary studies on toxic cyanobacteria in India and for the first time provide evidence of the occurrence of MCYSTs in toxic water blooms. The results also support the hypothesis of the co-existence of MCYSTs and nontoxic peptides, particularly those inhibiting animal proteases [17]. MCYSTs were detected in 20% of the *Microcystis* blooms in the current study area, with an abundance of MCYST-RR. Interestingly, in an earlier study, these blooms also exhibited toxicity to the cladoceran *M. macrocopa* [1]. Thus, the toxicity to zooplankton was apparently attributable to the presence of MCYSTs, although this hypothesis needs further verification.

The concentration of MCYSTs in the natural samples varied considerably, and was primarily due to the species of *Microcystis* in the blooms. Among the various commonly occurring species of *Microcystis*, MCYSTs have been detected most frequently in *M. aeruginosa*, whereas low proportions of *M. viridis* and no *M. wesenbergii* colonies have been shown to be MCYST producers [30]. Consequently, it was recently hypothesized that the sporadic distribution of MCYST producers either between or within genera is the result of repeatedly losing the ability to produce MCYSTs in the course of evolution [23].

To some extent, environmental factors also affect the net amount and composition of MCYSTs. For example, conditions that favor optimal cell division rates, such as high phosphorus concentrations, accelerate MCYST production [20], and more demethylated derivatives of MCYSTs are produced under conditions of high temperatures, a high light intensity, and excess N-load [26]. Furthermore, it was recently found that for all morphospecies of *Microcystis*, both microcystin-producing and -nonproducing strains are present [35]. In another study on the distribution of *Microcystis* sp. in European freshwaters, similar morphospecies from different locations were found to contain different variants of microcystins [30]. In other words, several chemotypes with respect to their microcystin patterns can be expected in a morphospecies. The relative proportions of different variants and derivatives of MCYSTs may also affect the overall toxicity of the bloom. For example,

MYCST-RR is significantly less toxic than MCYST-LR, and demethylated derivatives are even lesser toxic [4, 10].

Accordingly, in this study also, the extent of toxicity varied significantly among the blooms comprised of a relatively similar abundance of the three morphospecies of *Microcystis*. The Kundam lake bloom material containing MCYST-LR was found to be more toxic than the other blooms, even though very high and similar amounts of MCYST-RR were present in both the Kundam and Gosalpur samples, suggesting that the presence of MCYST-LR is the main but not the sole factor behind acute toxicity. The toxicity of the Shahpura blooms was low, owing to a lower concentration of MCYST-RR. When the highly toxic bloom material from Kundam lake accumulates at the shore, the concentration of MCYST-LR can be as high as 2.8 mg/l [11]. The provisional guideline value established by WHO for MCYST-LR is 1 µg/l. Thus, accidental consumption of the bloom material or direct skin contact could represent a significant human health risk. Furthermore the incidence of cattle death and skin lesions in children is fairly common upon exposure to such waters. Consequently, this clearly reveals the need for chemically detailed comparative studies of Indian water resources, taking into consideration the difference in toxicity of structural variants of MCYSTs.

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