



Distribution of microcystin synthetase genes in filamentous cyanobacterial phytoplankton and production of microcystin in water samples collected from Eastern Madhya Pradesh, India

Prashant Chaturvedi, Trashi Singh and Suvendra Nath Bagchi*

Cyanobacterial Research Laboratory, Department of Post Graduate Studies and Research in Biological Science, Rani Durgavati University, Jabalpur, MP, India
snbagchi_in@yahoo.com

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Abstract

The important value index, which is sum of relative abundance, biovolume and frequency, was calculated for different cyanobacterial genera present in phytoplankton scum/mat material in ten water bodies located in three districts of Eastern Madhya Pradesh. All such scum/mat samples were dominated by cyanobacteria mostly belonging to the genera *Oscillatoria*, *Anabaena*, *Nostoc*, *Phormidium* and *Spirulina*. The microcystin synthetase genes (*mcyABDE*) were detected in all cyanobacterial scum/mats samples indicating that toxic genotypes of cyanobacteria constituted the populations. Despite ubiquitous presence, *mcy* genes displayed quite a patchy distribution pattern, and rarely all the four genes were present together. Present study showed amplification of *mcyA* (80%), *mcyB* (60%), *mcyD* (50%) and *mcyE* (80%) genes. The dissolved microcystin content in the waters harbouring cyanobacterial populations was determined by semi-quantitative ELISA. About 30% water bodies contained the free microcystins below 0.5 ppb, whereas the remaining ones showed presence of the toxin in a range of 0.5-3.0 ppb. These values are well below 1 $\mu\text{g L}^{-1}$ microcystin, a benchmark set for safe use of water for drinking and recreational purposes according to WHO guidelines.

Keywords: Biodiversity, Agarose gel electrophoresis, ELISA, Microcystin, PCR amplification.

Introduction

Microcystin is produced by bloom forming toxigenic cyanobacteria^{1,2}. Approximately 80 different variants of microcystins have been identified, being produced by different cyanobacterial genera. The commonest are the microcystin-leucine-arginine (LR), -arginine-arginine, (RR) and -tyrosine-arginine (YR) variants³. Microcystins are toxic in nature, which lead to impaired liver function and even fatality². Microcystins are synthesized by non ribosomal peptide/polyketide synthetase multi-enzyme complex⁴. Cyanobacteria falling under genera *Microcystis*, *Anabaena* and *Planktothrix* (*Oscillatoria*) are potentially known to produce microcystins^{5,6}. Though *Phormidium* and *Nostoc* are also considered as microcystins producing genera, they are rarely associated with profuse toxic bloom formation⁷⁻⁹. Microcystins are cyclic heptapeptides in which ADDA (3-amino-9-methoxy 2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) and D-Glu interact with and inhibit protein phosphatases and manifest toxicological responses^{10,11}. Almost all toxigenic filamentous cyanobacteria occurring in planktonic or benthos forms such as *Oscillatoria*, *Phormidium*, *Anabaena*, and *Nostoc* produce microcystin in the water system, and since the planktonic cyanobacteria frequently undergo lysis and decomposition¹², microcystins are released in water. Consequently, the toxin threatens the ecology and sustainability of aquatic system, affecting quality of drinking

water, irrigation, fishing, recreation and aquatic food products. Moreover, microcystin accumulating into food chain leads to many diseases in human and animals¹³⁻¹⁵. In this paper we investigated the diversity and dominance of filamentous cyanobacteria in different water bodies and determined the presence of *mcyABDE* genes by PCR amplification. Free microcystin concentration was also analyzed in waters by semi-quantitative ELISA technique.

Materials and methods

Chemicals: All general purpose chemicals were procured from HiMedia (India) and Sigma-Aldrich (USA). The primers for PCR amplification were purchased from Imperial Life Sciences Pvt. Ltd. (India) and ELISA kit purchase from Enviro Logix Inc. (USA).

Survey of study area and sampling locations: Ten permanent ponds, lakes and man-made reservoirs located in Jabalpur, Seoni and Dindori districts (79°E – 81°E longitude and 22°N – 24°N latitude) were surveyed, and during March 2014 - June 2015 cyanobacterial samples in the form of floating scum/mats were collected in wide mouth plastic bottles and brought to lab in ice box. The morphological features for identification up to the genus level were examined according to the keys as described by Desikachary¹⁶ at 10 X and 40 X magnifications of objective lenses (Olympus, Optical Corporation Ltd, Japan).

Determination of Important value index (IVI): For calculation of IVI, sum of the three indexes namely relative abundance relative frequency and relative bio-volume of each type genus of cyanobacteria was considered¹⁷. The maximum score that was 300 which is sum of maximum abundance (100%), frequency (100%) and bio-volume (100%) of all the individual genera present in cyanobacterial population. To calculate the abundance, enumeration of the cyanobacterial scum/mats was carried out according to Jayatissa et al¹⁷, by counting the number of cells and filaments present in 0.01 ml of cyanobacterial scum/mats sample carrying cyanobacterial genera using a Neubauer haemocytometer. For determination of abundance, 1.0 ml of scum/mats material pipette out from the scum/mats samples of the bottle and was added to 9.0 ml of distilled water and 10 μ l from it was placed on the surface of the slide platform. Frequency of individual genus of cyanobacteria was calculated according to the Jayatissa et al¹⁷. First of all 100 μ l of sample were dropped on a clean slide and smeared, which was covered with a large size cover slip without spilling the sample. Slide was observed under 40X objective lens (Olympus, Optical Corporation Ltd, Japan). Total number of individual cyanobacterial filaments specific for each genus was counted in the entire field to calculate frequency of individual cyanobacterial genus present in 1.0 ml of water sample. Relative frequency of each was calculated in percentage. Biovolume of cyanobacterial genera was determined by counting ocular divisions between the ends of filaments (cylinder) denoted as “h” and radius of the filament (cylinder) represented as “r”. All measurements were done in triplicate for filament and readings for three different filaments were taken and then calculated average size of each cyanobacterial genus. Biovolume was calculated using a formula, $\pi r^2 h$, and represented in mm^3 per liter of water.

DNA extraction: DNA extraction method of Jungblunt and Neilan¹⁸ adopted as “method 1” Ghosh et al¹⁹ was used for extracting genomic DNA from 25 mg lyophilized scum/mats. The material was heated at 65°C for 2 h in 3.0 ml of a DNA extraction buffer containing 800 mM ammonium acetate, 20 mM EDTA, 100mM Tris-HCl (pH 8.0), 1 % SDS and 1 % lysozyme (freshly prepared). Thereafter, 50 μ l of RNase from a stock of 10 mg ml^{-1} was added and further incubation was carried out at 37 °C for 30 min. To stop the reaction, mixture was chilled for 10 min at 4°C and centrifuged at 12000 \times g for 10 min at 4°C. To one volume of cell extract was added one volume of ice cold isopropanol and 0.1 volume of 4 M ammonium acetate and centrifuged at 12000 \times g for 10 min at 4°C to precipitate the DNA. Agarose gel electrophoresis was conducted for ascertaining the quality of DNA. Purity of DNA was also checked by taking the ratio of absorbance at A_{260}/A_{280} nm and DNA yield as $\mu\text{g ml}^{-1}$ of each sample was calculated by using the following formula: $A_{260} \times \text{dilution factor} \times 50$.

PCR amplification: PCR amplification of *mcyABDE* genes, preparation of reaction mixture and amplification cycles were carried out as described by Jungblunt and Neilan¹⁸ and Kumar et

al¹². A reaction mixture of 23 μ l was prepared using the PCR amplification kit containing: 12.5 μ l of autoclave distilled water, 2.5 μ l of 10 \times taq polymerase buffer, 1 μ l of 25 mM MgCl_2 , 4 μ l of 200 μ M of dNTPs mixture solution, 1 μ l of 20 pmol forward and reverse primers, 1 μ l of 1U taq DNA polymerase, 2 μ l of template DNA sample, 50 ng in each case. Temperature cycles were provided using Merck Genei thermocycler with specifications as follows: denaturation at 94°C for 5 min followed by 40 cycles of 94°C for 1 min each, then annealing at 60.8°C for 1 min each and extension at 72°C for 1 min each. This was followed by a final extension cycle of 10 min at 72°C. The amplified product was then analyzed using agarose gel electrophoresis. The primer sets used were as under:
mcyAF5'-AAAATTTAAAAGCCGTATCAAA-3'
mcyAR5'-AAAAGTGTTTTATTAGCGGCTCAT-3'
mcyBF 5'-CTATGTTATTTATACATCAGG-3'
mcyBR 5'-CTCAGCTTAACTTGATTATC-3'
mcyDF 5'-GATCCGATTGAATTAGAAAG-3'
mcyDR5'-GTATTCCCCAAGATTGCC-3'
mcyEF 5'-TTTGGGGTTAACTTTTTTGGGCATAGTC-3'
mcyER 5'-AATTCTTGAGGCTGTAAATCGGGTTT-3'

ELISA test for microcystins: Water samples were collected from each water bodies and brought to the laboratory. Thereafter, water samples were filtered with muslin cloth and nitrocellulose membrane filter using vacuum pressure. Filtration method used for as much as possible to remove finest particles present in water samples, as particles may chock ODS cartridge. The ODS-cartridges (Millipore Corporation, Bedford) were fitted on top with syringes using tubings and manual pressure was applied on the contents of the cartridges. Firstly, the cartridges were pre-washed with 10 ml of 100% methanol in order to equilibrate and subsequently they were washed with 10 ml of distilled water. Gradually the entire one liter water sample was passed through the cartridges. The bound material was first washed with 10 ml of 20% methanol which was discarded. Microcystins were eluted using 10 ml of 100% methanol.. The eluted 100% methanolic extract was kept for air drying for 1 to 2 days until methanol was evaporated. The dried matter was suspended in 1 ml of water containing 10% methanol and stored under refrigeration until used for ELISA test. The Quali Tube kit (Envirologix Inc., USA) was used for microcystin analysis. In this ELISA test were performed according to the manufacturer's instruction.

Results and discussion

Microscopic examination and identification of filamentous planktonic cyanobacteria: The filamentous cyanobacterium, *Oscillatoria* (Figure-1a) dominated the phytoplankton scums and mats of cyanobacteria comprising of multicellular cylindrical filament, either present singly or in the form of flat or spongy thallus, sheath was absent, motility mostly by creeping movement, filaments generally bent like a sickle or coiled more or less like a screw and filament size between 100 and 650 μ m.

Phormidium (Figure-1b) was also a major part of scum phytoplankton population comprising of leathery thallus, thin, bent cylindrical filament, pale blue-green filaments and colourless sheath and filament size between 260 and 600 μm .

Anabaena (Figure-1c) appeared generally in phytoplankton with broad uniform filament, long, cells generally barrel shaped, many heterocysts regularly present in a filament, and filaments size was between 115 and 300 μm .

Nostoc (Figure-1d) generally exhibited in cyanobacterial population with broad filament, cells generally spherical in shape, heterocysts intercalary and in young condition at terminal positions. Filaments size was between 110 and 350 μm .

Spirulina (Figure-1e) exhibited features such as thick multicellular filament, cylindrical, regularly loose or tightly coiled, and with large diameter and large spirals or comparatively short and fewer coils. There existed also few species of *Spirulina* filaments with less diameter and small spirals with comparatively large number of tight coiling. Filament size was between 150 and 350 μm (Figure-1f).

Diversity of filamentous planktonic cyanobacteria: Ten water bodies were examined for cyanobacterial diversity related investigation, surveyed during March 2014 to June 2015 for determination of composition of filamentous cyanobacteria. Present data show the mean value of relative biovolume and frequency of each filamentous genus of cyanobacteria as against such values corresponding to the total cyanobacterial population. We found exclusive *Oscillatoria* presence in Shahpura Lake (Dinori). Phytoplankton population in this study also revealed that the *Oscillatoria* was the major constituent dominantly present in all the lakes examined. The other forms of filamentous cyanobacteria were *Phormidium*, *Anabaena*, *Nostoc* and *Spirulina* that were present at sub-dominant level. Relative abundance for *Oscillatoria* generally was found to be between 85 – 95% and relative abundance for other forms were: *Phormidium* (44–75%), *Anabaena* (6–16%), *Nostoc* (5–14%) and *Spirulina* (5–11%). Relative frequencies were found for *Oscillatoria* between 64–88% and frequencies for other forms were found to be: *Phormidium* (29–60%), *Anabaena* (13–24%), *Nostoc* (5–14%) and *Spirulina* (5–11%) (Table-1).

As an exception, *Oscillatoria* sp. was present as a sub-dominant genus in Tonga Lake (Jabalpur) which exhibited comparatively low relative abundance (27%), in comparison to the other lakes. Accordingly, the relative frequency values in this lake was also low (40%) for *Oscillatoria*. On the other hand, *Phormidium* was found to be the dominant cyanobacterial phytoplankton constituent in the Tonga Lake (Jabalpur), where relative abundance (74%) and relative frequency (60%) for *Phormidium* were much higher than the corresponding values of *Oscillatoria*.

The total cyanobacterial biovolume of each water body is shown in Table-1. We found biovolume of *Oscillatoria* 8.50–48.22

mm^3L^{-1} , *Phormidium* 10.40–15.35 mm^3L^{-1} , *Anabaena* 13.65–28.15 mm^3L^{-1} , *Nostoc* 14.85 – 38.55 mm^3L^{-1} and *Spirulina* 4.21 – 5.45 mm^3L^{-1} .

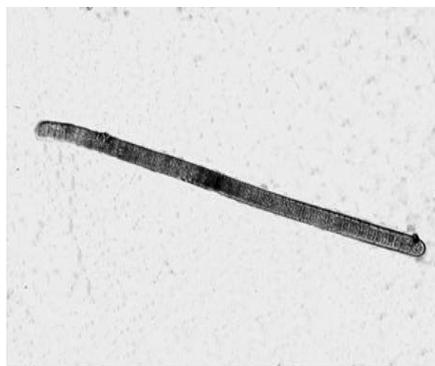
The Important Value Index (IVI) was calculated and presented in Figure-5. The IVI values was calculated based on a sum of relative abundance, frequency, and biovolume which clearly indicated that *Oscillatoria* was the major dominant constituent of planktonic cyanobacterial population in the examined water bodies; thereafter *Phormidium*, *Anabaena*, *Nostoc* and *Spirulina* were present as sub-dominant forms in the planktonic cyanobacterial population found in given water bodies.

Occurrence of microcystin producing *mcy* genes in scum/mats containing water sample: The recovery of DNA was between 80 – 100 $\mu\text{g ml}^{-1}$, as determined from ratio $A_{260/280}$ which was 1.52 – 1.63. Also prominent genomic DNA bands appeared underneath the wells of gel (Figure-3.) DNA material extracted from scum/mat samples collected from ten water bodies containing *Oscillatoria*, *Anabaena*, *Spirulina* and *Nostoc* was used for PCR amplification of *mcyABDE* genes. The specific primers used were highly effective for the detection of *mcy* genes present in the planktonic cyanobacteria. Positive amplification indicated that constitution of planktonic cyanobacterial genera carrying microcystin toxin producing genes and termed as *mcy*+ve genotypes. These planktonic cyanobacteria recovered in this investigation were presumably capable of producing microcystin as a large majority of them were *mcy*+ve genotypes. The *mcyA* gene was amplified in eight (80%), *mcyB* in six (60%) whereas *mcyE* in eight (80%) water bodies. The *mcyA*, *mcyB* and *mcyE* genes amplicon size corresponded to ca. 295, 800 and 472 bp respectively (Figure-4). However with regard to *mcyD* specific primers, the characteristic amplicon size of about 818 bp was discernable only in five out of ten samples of cyanobacteria containing scum/mats (Figure-4).

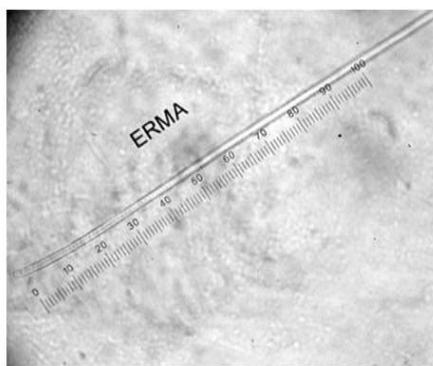
All four *mcy* genes amplified in Paharikhera Lake (Jabalpur) and Shankarmadia Lake (Seoni). Only *mcyE* gene was found to be present in Babariya Lake (Seoni) samples and only *mcyD* gene was detected in Samnapur Lake (Jabalpur) samples.

Microcystin production in water samples harbouring *mcy*+ genes of cyanobacteria: Cell-bound microcystin upon decay of filamentous cyanobacterial scum/mats is released in the water as free toxins.

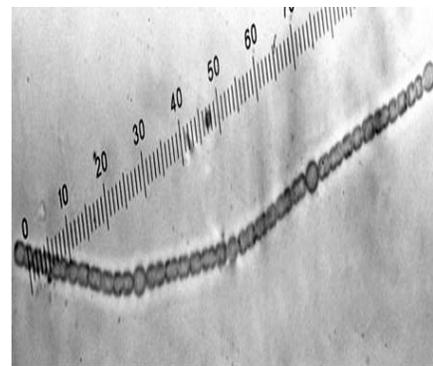
All *Oscillatoria* predominant scum/mats samples analyzed showed amplification of *mcy* genes, thus such scum/mats can be considered as potentially toxigenic cyanobacteria, releasing microcystins in the adjoining water. We determined the free microcystin concentration inside water by semi quantitative ELISA test of the ten water bodies, which were infested with cyanobacteria. It is clear from Table-2 that seven (70%) water samples contained free microcystin concentration between 0.5 ppb and 3.0 ppb and the rest of the 30% water samples showed below 0.5 ppb of free microcystin.



(a) *Oscillatoria* sp.



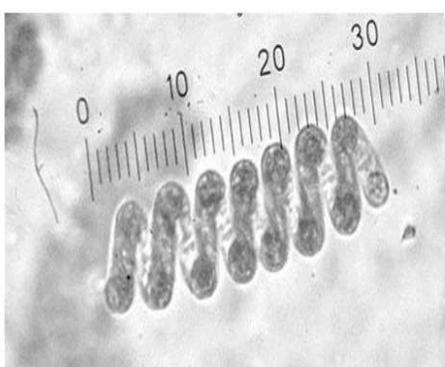
(b) *Phormidium* sp.



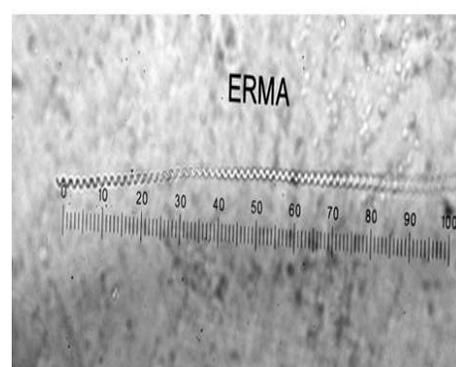
(c) *Anabaena* sp.



(d) *Nostoc* sp.



(e) *Spirulina* sp.



(f) *Spirulina* sp.

Figure-1: Microscopic images of cyanobacterial genera present in scum/mats samples collected from different water bodies of Eastern Madhya Pradesh (India).

Table-1: Percentage frequency and mean biovolume of cyanobacterial populations of selected water bodies of Eastern Madhya Pradesh

Name of water bodies		Cyanobacterial frequencies (%)	Mean biovolume $\text{mm}^3 \text{L}^{-1}$
1.	Shankarmadia lake, Seoni	<i>Oscillatoria</i> (76), <i>Anabaena</i> (24)	36.7
2.	Babariya lake, Seoni	<i>Oscillatoria</i> (80), <i>Anabaena</i> , (20)	52.2
3.	Paharikhera lake, Jabalpur	<i>Oscillatoria</i> (63), <i>Phormidium</i> (29), <i>Spirulina</i> (8)	50.6
4.	Tonga lake, Jabalpur	<i>Oscillatoria</i> (40), <i>Phormidium</i> (60)	39.9
5.	Dalsagar lake, Seoni	<i>Oscillatoria</i> (88) <i>Spirulina</i> (12)	16.3
6.	Bahela lake, Jabalpur	<i>Oscillatoria</i> (64) <i>Nostoc</i> (36)	22.8
7.	Kundam lake, Jabalpur	<i>Oscillatoria</i> (84) <i>Nostoc</i> (16)	87.7
8.	Shahpura lake, Dindori	<i>Oscillatoria</i> (100%)	8.5
9.	Samnapur lake, Jabalpur	<i>Oscillatoria</i> (68), <i>Anabaena</i> (32)	33.4
10.	Gangasagar lake, Jabalpur	<i>Oscillatoria</i> (68), <i>Nostoc</i> (32)	25.4

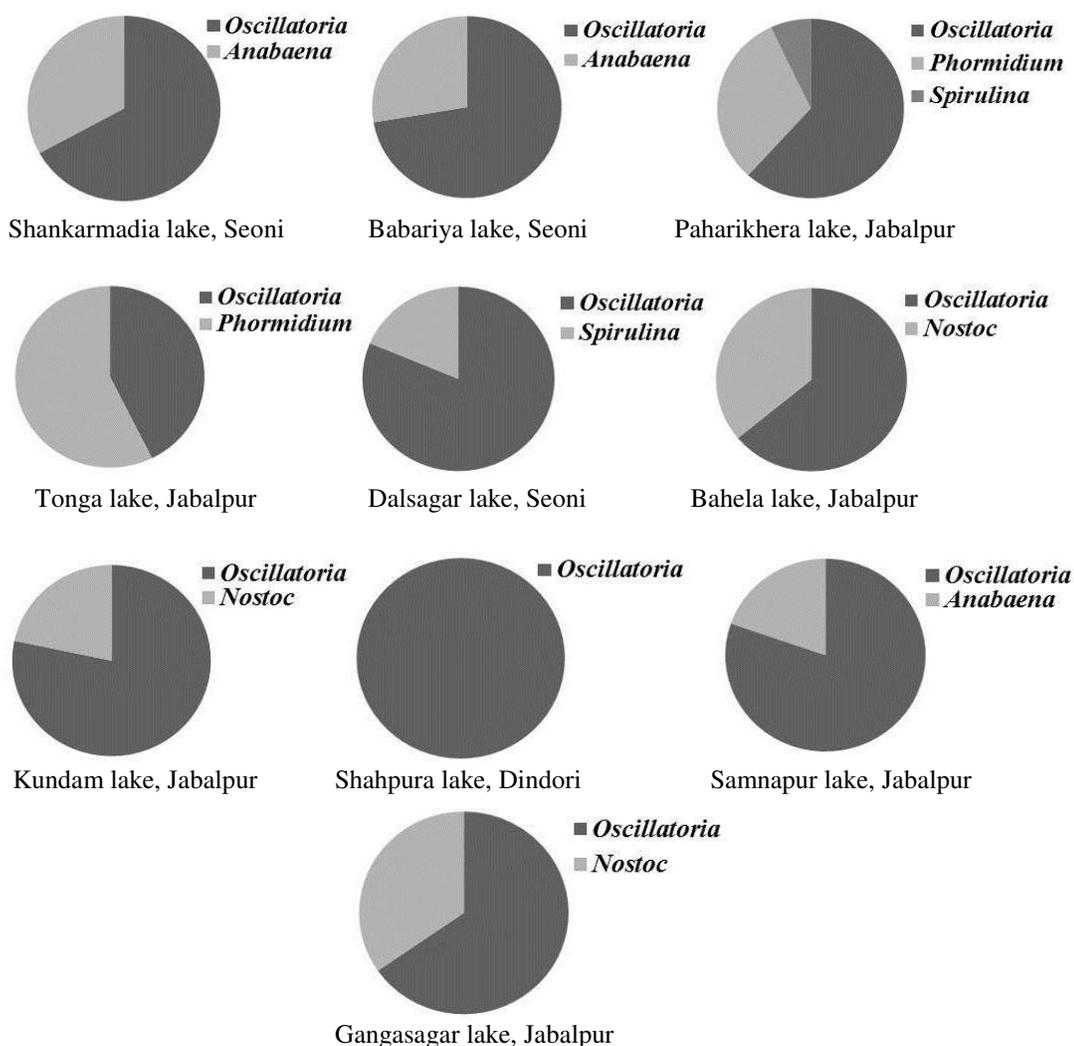


Figure-2: Important value index (IVI) of filamentous cyanobacterial community present in samples of different water bodies collected from Eastern Madhya Pradesh (India).

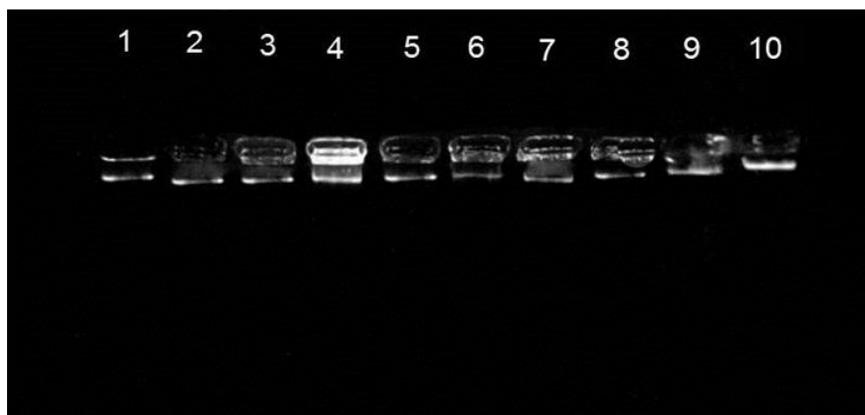


Figure-3: Agarose gel electrophoresis of DNA isolated from cyanobacterial scum/mats samples (1) Shankarmadia lake, Seoni, (2) Babariya lake, Seoni, (3) Paharikhera lake, Jabalpur, (4) Tonga lake, Jabalpur, (5) Dalsagar lake, Seoni, (6) Bahela lake, Jabalpur, (7) Kundam lake, Jabalpur, (8) Shahpura lake, Dindori, (9) Samnapur lake, Jabalpur, (10) Gangasagar lake, Jabalpur.

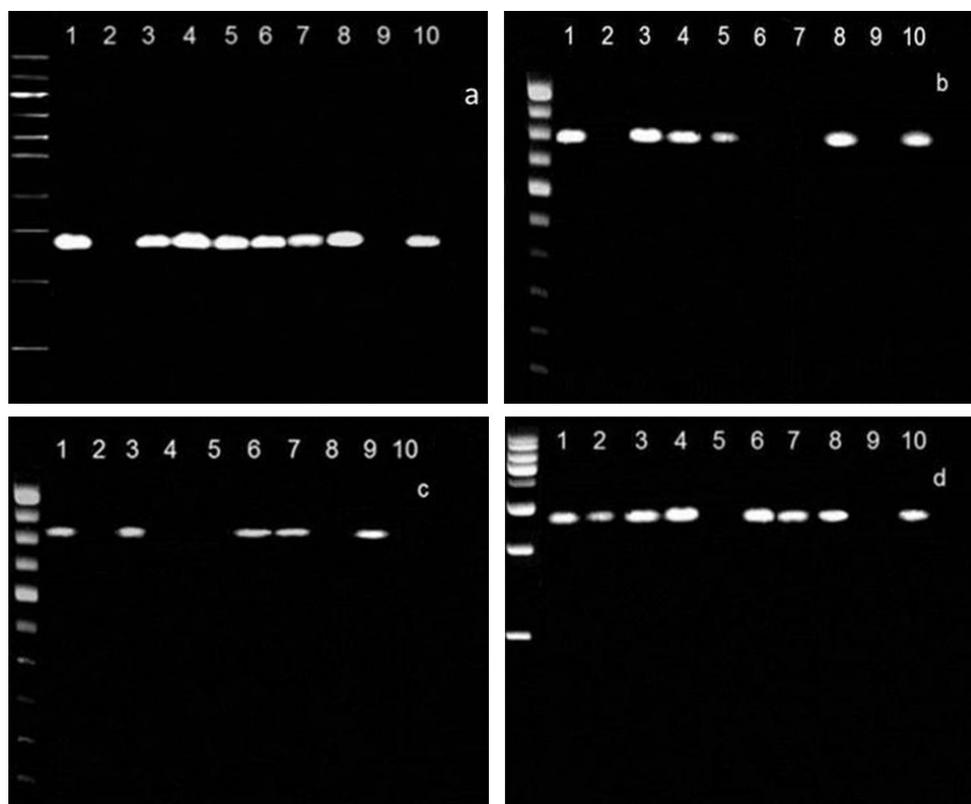


Figure-4: The prevalence of *mcy*+ve genotypes by virtue of presence of (a) *mcyA*, (b) *mcyB*, (c) *mcyD* and (d) *mcyE* genes were resolved at 295, 800, 818 and 472 bp, respectively. Lane 1 – 10 sequences similar to Fig – 3 Agarose gel electrophoresis of DNA from cyanobacterial scum/mats samples.

Table-2: Levels of free microcystins in the ten water bodies of Eastern Madhya Pradesh.

Name of water bodies	Microcystin concentration range in water bodies
1. Shankarmadia lake, Seoni	≥ 0.5 ppb ; ≤ 3.0 ppb
2. Babariya lake, Seoni	≤ 0.5 ppb
3. Paharikhera lake, Jabalpur	≥ 0.5 ppb ; ≤ 3.0 ppb
4. Tonga lake, Jabalpur	≥ 0.5 ppb ; ≤ 3.0 ppb
5. Dalsagar lake, Seoni	≤ 0.5 ppb
6. Bahela lake, Jabalpur	≥ 0.5 ppb ; ≤ 3.0 ppb
7. Kundam lake, Jabalpur	≥ 0.5 ppb ; ≤ 3.0 ppb
8. Shahpura lake, Dindori	≥ 0.5 ppb ≤ 3.0 ppb
9. Samnapur lake, Jabalpur	≤ 0.5 ppb
10. Gangasagar lake, Jabalpur	≥ 0.5 ppb ; ≤ 3.0 ppb

Discussion: In this study, we have determined three separate parameters: relative abundance, frequency and biovolume that gives an idea about biodiversity of cyanobacteria. These parameters were combined to establish IVI, which is an accurate analysis to demonstrate the relative dominance of cyanobacteria in scum/mats. The IVI value was proven to be advantageous over solely abundance, biovolume or frequency as an independent parameter in determining the relative composition of cyanobacteria. Based on IVI values, *Oscillatoria* spp. were found to be present dominantly in water bodies whereas other filamentous cyanobacterial spp preferentially co-habited as sub-dominant forms with *Oscillatoria*. There has been a shift of cyanobacterial population from *Microcystis* spp. as reported in previous surveys^{19,20} to *Oscillatoria*, *Anabaena*, *Nostoc* and *Spirulina* containing phytoplankton in Kundam and Seoni towns, e.g. Dalsagar and Kundam lakes (cf. Table-1). Transition of water bodies from high level of nitrate and phosphate to high phosphate and low nitrate encouraged appearance of nitrogen-fixing forms. Owing to periodic recycling of water in these water bodies and better sunlight penetration in deeper water columns, *Oscillatoria* grew abundantly and floated as scums/mats replacing the previous *Microcystis* abundant populations. This succession could also be partially due to an algicidal effect of *O. laetevirens*, a perennial cyanobacterium present as a phytoplankton in these waters²¹.

PCR amplification of *mcyABDE* genes (cf. Figure-4) in the scum/mats samples of predominant *Oscillatoria* collected from different water bodies revealed sporadic distribution of *mcy* genes. Possibly these samples contain incomplete *mcy* gene clusters. One can assume that the *mcy* genes were rendered non-functional due to random mutational hits on the genome arising from deletion and recombination events in the genomic sequences²². Earlier it was reported that filamentous cyanobacteria carry *mcy* gene cluster, and *Oscillatoria* and *Phormidium*, *Anabaena*, and *Nostoc* are perhaps toxigenic genera^{12,19}. About 70% of all the population showed microcystin production in a range between 0.5 and 3.0 ppb and for 30% samples it was below 0.5 ppb. The investigated water bodies showed negligible free microcystins, and by WHO recommendations it is too low to impose any health problems²³. Compiling the study reports^{13,19,24}, it appears that microcystin producing *Oscillatoria* genotypes are more successful to proliferate than the other filamentous forms. We do not rule out the presence of microcystins or other cyanotoxins, viz anatoxin in the sub-dominant cyanobacteria such as *Anabaena* sp.

Conclusion

Eastern Madhya Pradesh (India) is a tropical zone and highly polluted and eutrophic water bodies are located in this area. These water bodies provide favorable conditions for the planktonic cyanobacteria, and few toxic cyanobacterial genera grow profusely. Toxic cyanobacteria contain microcystin synthetase (*mcy*) gene cluster in their genomic DNA. These toxic cyanobacterial genera are present in almost all scum/mats, and biofilms. All scum/mat samples were *Oscillatoria* dominant and other genera present as subdominant forms. A majority of toxigenic cyanobacteria also released free microcystins in the surrounding waters whose levels were 0.5 - 3.0 ppb or was below 0.5 ppb.

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