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Full Length Research Paper

PCR-based detection of *mcy* genes in blooms of *Microcystis* and extracellular DNA of pond water

Anil Kumar[1], Ashok Kumar[1]*, Ashutosh Kumar Rai[1] and Madhu Bala Tyagi[2]

[1]School of Biotechnology, Banaras Hindu University, Varanasi-221 005, India.
[2]Department of Botany, MMV, Banaras Hindu University, Varanasi-221 005, India.

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Microcystin from *Microcystis aeruginosa* is the most widely studied hepatotoxin which is synthesized by the ~55-kb microcystin synthetase (*mcy*) gene cluster. In this study, employing polymerase chain reaction (PCR) amplification of six genes of the *mcy* genes cluster (*mcyA*, *mcyB*, *mcyC*, *mcyD*, *mcyE* and *mcyG*), the presence of the toxic cyanobacterium, *Microcystis* has been demonstrated in seven eutrophicated ponds of Varanasi. Unlike the DNA recovered from blooms or whole cells, extracellular DNA present in pond water was used as template which showed amplification of all the desired *mcy* genes. Additionally, amplification of five genes namely, *mcyB, C, D, E* and *G* was noted in a single reaction by multiplexing of desired primers. Our findings suggest that, (i) extracellular DNA present in water may be directly used as template, and (ii) multiplex PCR may be routinely employed for the monitoring of *mcy* genes. This study seems important especially for those ponds where blooms of *Microcystis* may be visibly absent but water does contain microcystin-LR. To our knowledge detection of *mcy* genes in any pond water using extracellular DNA as template in PCR assay has not been reported so far.

Key words: *Microcystis,* cyanobacteria, microcystins, pond water, multiplex-PCR, extracellular DNA.

INTRODUCTION

Since the first report of toxic cyanobacteria (*Nodularia spumigena*) in the late 19th century, toxic cyanobacterial blooms have been reported from many parts of the world (Carmichael, 1994; Oberholster et al., 2004; Kurmayer and Christiansen, 2009). Toxins of cyanobacteria (cyanotoxins) are very diverse in their chemical structure and toxicity (Kaebernick and Neilan, 2001). Several cyanobacterial toxins with hepato-, neuro- and dermatotoxic effects have been isolated and characterized (Tyagi et al., 1999; Kurmayer and Christiansen, 2009). Of these, microcystins (hepatotoxin) are the most ubiquitous cyanotoxins that have been responsible for the widespread deaths of animals throughout the world (Carmichael, 1994; Oberholster et al., 2004). Microcystins are cyclic heptapeptides having variable amino acids at position 2 and 4. More than 89 structural variants are known and many species produce several microcystin variants, but only one or two isoforms dominate in any individual species (Pearson and Neilan, 2008). Microcystin is synthesized nonribosomally by the microcystin synthetase enzyme complex through a thio-template mechanism (Tillett et al., 2000; Pearson and Neilan, 2008). The gene cluster encoding microcystin synthetase enzyme complex, *mcy* has been sequenced and characterized in detail from *Microcystis*, *Planktothrix* and *Anabaena* (Nishizawa et al., 2000; Tillett et al., 2000; Christiansen et al., 2003; Rouhiainen et al., 2004; Kurmayer and Christiansen, 2009).

In *Microcystis aeruginosa,* the *mcy* gene cluster comprises 10 genes arranged in two divergently transcribed operons, *mcyA-C* and *mcyD-J* (Kaebernick et al., 2002; Pearson and Neilan, 2008).

In general, screening and detection of *Microcystis* from

*Corresponding author. E-mail: kasokbt@rediffmail.com. Tel: +91- 542- 2368331/6701593. Fax: +91 -542 -2368693.

bloom-infested water had been made mainly on microscopic techniques combined with the chemical detection of microcystin in the water samples (Paerl, 1988; Kumar et al., 2000). However, differentiating toxin-producers among cyanobacterial species/strains based on morphological features of the cells are beyond reach. After the elucidation of *mcy* genes cluster, several studies have applied molecular methods for monitoring the presence of harmful toxic cyanobacteria and the genes involved in the biosynthesis of cyanotoxins (Ouahid et al., 2005; Rantala et al., 2006; Hotto et al*.,* 2007; Saker et al., 2007; Pearson and Neilan, 2008; Valério et al., 2010). Recently, Oberholster and Botha (2010) have applied remote sensing and molecular markers comprising *mcyA, B* and *D* genes to detect toxic cyanobacterial hyperscum crust from South Africa. In general, amplification of *mcy* genes by polymerase chain reaction (PCR) has been performed using whole cells/filaments or colonies/crusts and genomic DNA extracted from the cells (Pan et al., 2002; Ouahid et al., 2005). No report exists on the use of extracellular DNA present in water for PCR assay. Since the blooms frequently undergo lysis and decomposition, some DNA is inevitably released in the water. In our preliminary study we detected the presence of DNA in water of several ponds. This aroused our interest to screen the presence of toxic *Microcystis* directly from the extracellular DNA of pond water employing PCR-based amplification of *mcy* genes. Accordingly, we selected six *mcy* genes (involved in microcystin biosynthesis) and employed multiplex PCR to predict the presence of toxic *Microcystis* in the pond water.

MATERIALS AND METHODS

Blooms-collection, isolation of cyanobacteria and culture conditions

Seven ponds namely, Durgakund (DK), Lakshmikund (LK), Pishachmochan (PM), Kandawa (Kd), Laatbhairov (LB), Adityanagar (AN) and Guradari (Gd) which were found heavily infested with blooms of cyanobacteria either in summer months or throughout the year were selected for this study. A pond situated in the campus of diesel locomotive workshop (DLW), Varanasi, with no known history of cyanobacterial blooms was chosen as control site. Blooms were examined microscopically for ensuring the presence of cyanobacteria in all the samples. Based on microscopic observations, the cyanobacterial genera present in the bloom samples were identified. Different species of cyanobacteria present in the samples were isolated and grown as unialgal culture in BG11 or Parker's medium employing standard microbiological methods (Kumar et al., 2000).

Preparation of crude extract and toxicity test

250 ml of the bloom samples from all the ponds were collected with zooplankton net (100 µm mesh) during the summers (March to June) of 2006 and 2007 in sterilized glass bottles and brought to the laboratory. Bloom samples were lyophilized before preparation of crude extract. Mice bioassay for toxicity test was done as described by Kumar et al. (2000). Autopsy of sacrificed mice was done to examine gross signs of poisoning in the internal organs such as liver.

Estimation of microcystin

250 ml water samples (50 ml each from four corners and centre of the pond) from each pond were collected from a depth of 60 cm in an acid washed glass bottle and passed through zooplankton net (100 µm mesh) so as to minimize the presence of cyanobacterial cells/colonies. Thereafter water samples were filtered through Whatman No. 1 filter paper to remove particulate materials. The filtrates were centrifuged at 15,000 *g* for 10 min and filtered over membrane filter (0.20 µm pore size, Millipore Corp., USA). Such filtered water was used for the estimation of microcystin and extraction of extracellular DNA. Spreading of filtered water on to solid or liquid Parker's media did not show growth of any organisms suggesting complete removal of live cells from water by filtration processes. Amounts of microcystin-LR in pond water and extracts of unialgal cultures of cyanobacteria were determined by ELISA kit according to instructions of the manufacturer (Envirologix Inc., Portland, USA).

DNA isolation

Genomic DNA from the blooms and unialgal culture of *Microcystis* was isolated using DNeasy Tissue kit (Qiagen, Germany). Briefly, 50 to100 µl frozen cell suspensions were suspended in 270 µl ATL buffer. 30 µl proteinase K (20 mg/ ml) was added and mixed well. After incubation at 55°C for 5 to 6 h, the content was centrifuged at 5000 g for 3 min. Above steps resulted into complete lysis of the cells. The supernatant was transferred to a 1.5 ml eppendorf tube and 300 µl AL buffer was added and after proper mixing incubated at 70°C for 10 min. The pH of the lysate was adjusted to ~ 6 with 1 N HCl. Thereafter 300 µl of absolute alcohol was added and mixed well. The whole content was loaded on to the mini column and spun at 6000 g for 1 min. Further processing was done according to instructions of manufacturer. The DNA was eluted with 30 µl of elution buffer (AE).

Nucleic acids present in pond water were also recovered using the DNeasy Tissue kit. 25 ml of absolute alcohol was added to 50 ml filter sterilized pond water (part of those used for microcystin estimation) and mixed well. The solution was transferred to mini column and centrifuged at 6000 g for 1 min. All other isolation steps were similar to those described above. The purity of the DNA was checked by calculating the ratio between A_{260} and A_{280} (pure DNA preparations have A_{260}/A_{280} value of 1.8) in a Genova UV-Visible Spectrophotometer (Jenway, UK).

Amplification of the *mcy* genes

Amplification of *mcyA*, *mcyB*, *mcyC*, *mcyD*, *mcyE*, and *mcyG* was done from the genomic DNA extracted from bloom samples, unialgal culture of *Microcystis* and extracellular DNA recovered from water of different ponds. Sequences of primer sets used were adopted from Ouahid et al. (2005) and purchased from Integrated DNA Technologies, Inc., USA. Reaction was performed in a total volume of 50 µl which contained; 31.5 µl sterile Milli Q water, 5.0 µl 10 X *Taq* DNA polymerase assay buffer (Bangalore Genei, Bangalore, India), 2.0 µl 25 mM $MgCl_2$, 1.0 µl each (200 µM final concentration) dNTPs (Bangalore Genei), 1.0 µl each (10 pmol) of the forward and reverse primers, 20 ng template DNA and 0.5 µl

Table 1. Presence of cyanobacteria other than *Microcystis* and microcystin and nucleic acid content in various ponds water.

Ponds[a]	Other cyanobacteria	pH of water (May 2006)	Microcystin-LR content (µg/l)[b]	Toxicity of blooms[c]	Extracellular nucleic acid in water (ng/ml)
Durgakund	*Planktothrix, Spirulina Anabaenopsis*	8.36±0.26	1.20 ± 0.09	+	11.44 ± 0.29
Lakshmikund	*Cylindrospermum, Spirulina, Raphidiopsis*	9.34±0.21	1.30 ± 0.08	+	11.08 ± 0.29
Pishachmochan	*Planktothrix, Spirulina*	9.82±0.19	0.32 ± 0.05	+	15.24 ± 0.44
Kandawa	*Planktothrix, Plectonema*	8.82±0.23	0.71 ± 0.06	+	8.46 ± 0.34
Laatbhairov	*Spirulina, Lyngbya, Phormidium, Planktothrix,*	6.90±0.38	0.25 ± 0.07	+	4.12 ± 0.13
Adityanagar	*Cylindrospermum, Spirulina*	9.60±0.16	1.00 ± 0.04	+	12.62 ± 0.77
Guradari	*Phormidium, Planktothrix,*	7.40±0.29	0.20 ± 0.05	+	10.36 ± 0.21
DLW	None	8.62±0.24	Nd	-	8.60 ± 1.07

[a] Bloom samples from different corners of an individual pond were collected during the summers (March to June) of 2006 and 2007. All the bloom samples showed dominance of *Microcystis*. Water surface temperature ranged from 30-40^{0}C during the time of sampling.[b] Quantity of microcystin and nucleic acid is based on the average of four samples collected from an individual pond. Insignificant differences in the quantity were noted in the samples collected in 2006 and 2007. nd- not detectable. Laboratory-grown *Microcystis* isolated from the blooms of different ponds showed 6.5-7.0 µg microcystin-LR/mg protein. [c] 0.5 ml crude extract (approx. 1 mg protein/ml) of each bloom sample or laboratory – grown cultures of various cyanobacteria and 0.5 ml filtered water samples of various pond was used separately for toxicity assessment and response was recorded over 6 h. Results are based on three experiments conducted separately but under identical conditions. + (toxic), and – (non-toxic).

(1.5 U) *Taq* DNA polymerase (Bangalore Genei). PCR was run in a PTC-100 thermal cycler (MJ Research, USA). Thermal cycle for *mcy*A was set as described by Tillett et al. (2001). For the amplification of *mcyB, C, D, E* and *G* genes, thermal cycles were set according to Quahid et al. (2005). 5 µl of each amplified product was electrophoresed on an agarose gel (1.5% w/v) in TAE buffer at 50 V for 4 to 5 h. Gels were visualized after staining with ethidium bromide (0.5 µg/ml) and photograph was taken in a Gel Documentation System (Bio-Rad Laboratories, USA).

Multiplex PCR

Knowing that primer pairs for *mcyB*, *C*, *D*, *E* and *G* genes have identical annealing temperature (52°C), multiplex PCR was carried out in a single tube with the primers for respective genes. Thermal cycle for multiplex PCR was similar to those described above (Ouahid et al., 2005). 7.5 µl of the amplified PCR product was used for electrophoresis and visualization was made as mentioned above.

Homology of *mcy*A gene

For analyzing homology among *mcyA* gene, 1 µg amplified fragment (quantified by Nanodrop spectrophotometer) from the blooms of different ponds was digested with *Alu*I (2.5 U) and *Rsa*I (2.5 U) (New England Biolabs, USA) for 16 h at 37°C followed by inactivation at 65°C for 20 min in a water bath. The DNA fragments obtained were used to generate similarity index using NTSYSpc 2.1. For sequencing, *mcyA* gene from the bloom sample of DK pond was purified using Amicon Microcon-PCR Centrifugal Filter Devices (Millipore Corp., USA) following manufacturer's instructions. Purified *mcyA* amplicon was cloned into pGEM®-T Easy vector using cloning kit (Promega Corp., USA) and the insert was sequenced by the dideoxy-chain termination method using T7 and SP6 primers of the vector region by an automated DNA sequencer (ABI Prism; Model 3730). The *mcyA* sequence was compared with the GenBank database using the algorithm BLASTN programme to identify the most similar *mcyA*.

RESULTS

Abundance of blooms in various ponds

First we conducted routine survey of various ponds (n-18) around Varanasi city so as to assess the abundance of the blooms. Routine survey showed extensive development of blooms predominantly during the summers (March to June) of both 2006 and 2007 in seven ponds namely, DK, LK, PM, Kd, LB, AN and Gd. However, in certain ponds (DK, LK and AN) blooms were noticed throughout the year. Microscopic examination of the blooms revealed the dominance of *Microcystis* (mainly *M. aeruginosa)* but the presence of a few

cyanobacterial genera (in low number) was also noted (Table 1). However, occurrence of unicellular forms such as *Synechococcus, Gloeocapsa, Synechocystis* etc. was not visible in any of the bloom samples. pH of water of all the seven ponds was found in the range of 6.90 to 9.60 in the month of May 2006 (Table 1).

Toxicity of blooms/ laboratory-grown cultures

Intraperitoneal injection (i.p.) of crude extracts from the bloom samples and laboratory-grown unialgal culture of *Microcystis* of all the seven ponds killed the test animals within 1 h with a LD_{50} of 40 to 60 mg/kg body weight. Upon autopsy, the liver showed an enlargement and the colour changed to a deep red probably due to hemorrhaging and blood pooling. The toxicity symptoms resembled to those caused by typical hepatotoxic *M. aeruginosa* strains. However crude extracts of laboratory-grown unialgal cultures of other cyanobacterial genera including *Anabaenopsis, Cylindrospermum*, *Lyngbya*, *Plectonema, Planktothrix*, *Phormidium, Raphidiopsis* and *Spirulina* and filtered water samples failed to show toxicity.

Microcystin-LR content

Cell extracts of all the laboratory-grown cultures of cyanobacteria excluding *Microcystis* did not show detectable level of microcystin by ELISA kit. On the other hand laboratory-grown *Microcystis* cells routinely showed micorcystin-LR in the range of 6.5 to 7.0 µg/mg protein. Analysis of water samples of different ponds showed the presence of microcystin-LR in the range of 0.20 to 1.30 µg/l (Table 1).

Amplification of *mcy* genes

Bloom samples of all the seven ponds showed excellent amplification of *mcyA*, *B*, *C*, *D*, *E* and *G* genes (Figure 1). The size of various *mcy* amplicons matched with the size reported for other *Microcystis* spp. That the blooms of all the seven ponds probably contain closely related strain of *Microcystis* was evident from the morphological characters of the colonies as well as similarity in restriction digestion pattern of *mcyA* fragment (data not shown). Anticipating homology among *mcyA* gene of blooms of all the seven ponds, sequencing of *mcyA* from bloom sample of one representative pond (DK) was done. Analysis of the *mcyA* sequence (accession number DQ436931) showed 98% homology to *mcyA* sequence of *M. aeruginosa* (accession No.AB019578.2) available in the GenBank data base.

Amplification of *mcy* genes from extracellular DNA of pond water

After demonstrating the amplification of *mcy* genes from the genomic DNA recovered from the blooms, we focused our attention to test the applicability and usefulness of extracellular DNA present in water of various ponds as template for the amplification of *mcy* genes in a culture-independent approach. That the release of cellular constituents indeed occurs was evident from the estimation of microcystin-LR and DNA / nucleic acid content in water samples of various ponds (Table 1). Varying amount of extracellular nucleic acids (4.12-15.24 ng/ml of water) was noted in water of different ponds (Table 1). That the release of DNA from *Microcystis* cells indeed occurs became evident also from the amplification of six *mcy* genes from the DNA of pond water. Results obtained were identical to those obtained with the genomic DNA extracted from the blooms (Figures 2A and B). Absence of amplification from nucleic acids of LB is intriguing and could be due to very low concentration of *mcy* specific DNA fragments. As expected amplification was missing from the nucleic acids of DLW pond, water of this pond never showed the presence of *Microcystsis* and as such has no history of cyanobacterial blooms (Figures 2A and B).

Multiplex PCR for *mcy* genes

Although we successfully demonstrated the amplification of *mcyA*, *B*, *C*, *D*, *E* and *G* genes individually both from blooms and extracellular DNA of pond water, steps involved are lengthy and relatively expensive. As an alternative we adopted a protocol based on multiplex PCR assay knowing the fact that most of the primers used had annealing temperature at 52°C. From the gel photograph (Figure 2C), it is evident that multiplex PCR assay effectively amplified the five desired *mcy* genes from the extracellular DNA of various ponds. The size of all the five *mcy* genes was identical to those where amplification was performed individually using a single set of primers (Figures 2A and B).

DISCUSSION

Dominance of toxic cyanobacterial blooms in eutrophic lakes, rivers, ponds, and reservoirs has been reported from all over the world (Carmichael, 1994; Tyagi et al., 1999; Oberholster et al., 2004; Ouellette et al., 2006). Results of this study also revealed the abundance of blooms comprising *Microcystis* in all the seven ponds during summer months. In general, extensive development of blooms occurs during summer (Rinta-Kanto and Wilhelm, 2006; Saker et al., 2007), while it is

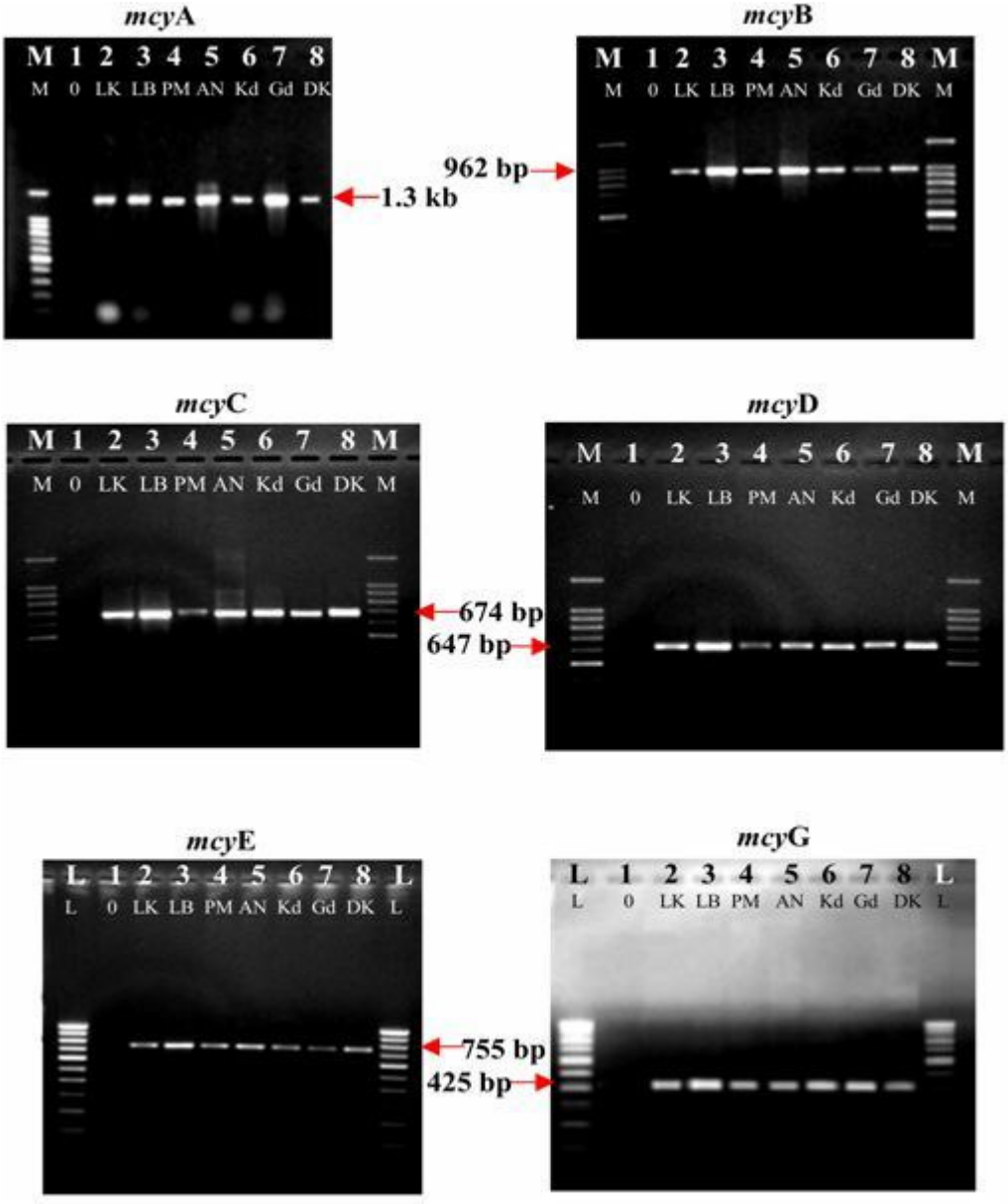

Figure 1. Amplification of *mcy* genes (*mcyA*, *B*, *C*, *D*, *E* and *G*) from the blooms of different ponds infested by *Microcystis.* Lane: 1- control (without template DNA); lanes 2-8 correspond to *mcy* gene amplified from: 2- Lakshmikund (LK); 3- Laatbhairov (LB); 4- Pishachmochan (PM); 5- Adityanagar (AN); 6- Kandawa (Kd); 7- Guradari (Gd); and, 8- Durgakund (DK). Lanes M and L, contain 100 bp DNA ladder.

found at low concentrations in rest seasons of the year. Occurrence of blooms throughout the year in certain ponds in this study may be attributed to high level of eutrophication and pH of water. Additionally, higher temperature prevailing in the tropical region might also be responsible for the development of blooms. Besides *Microcystis* species, several studies have demonstrated synthesis of microcystins by a range of cyanobacterial species including *Anabaena*, *Nostoc*, *Anabaenopsis, Hapalosiphon*, and *Planktothrix* (Rouhiainen et al*.,* 2004; Kurmayer and Christiansen, 2009).

Evidently, none of the bloom samples in this study contained *Anabaena* or *Nostoc* and other cyanobacteria were present in small numbers, obviously the sole microcystin-producing species seems to be the species of *Microcystis*. Most probably the strains of *Planktothrix*

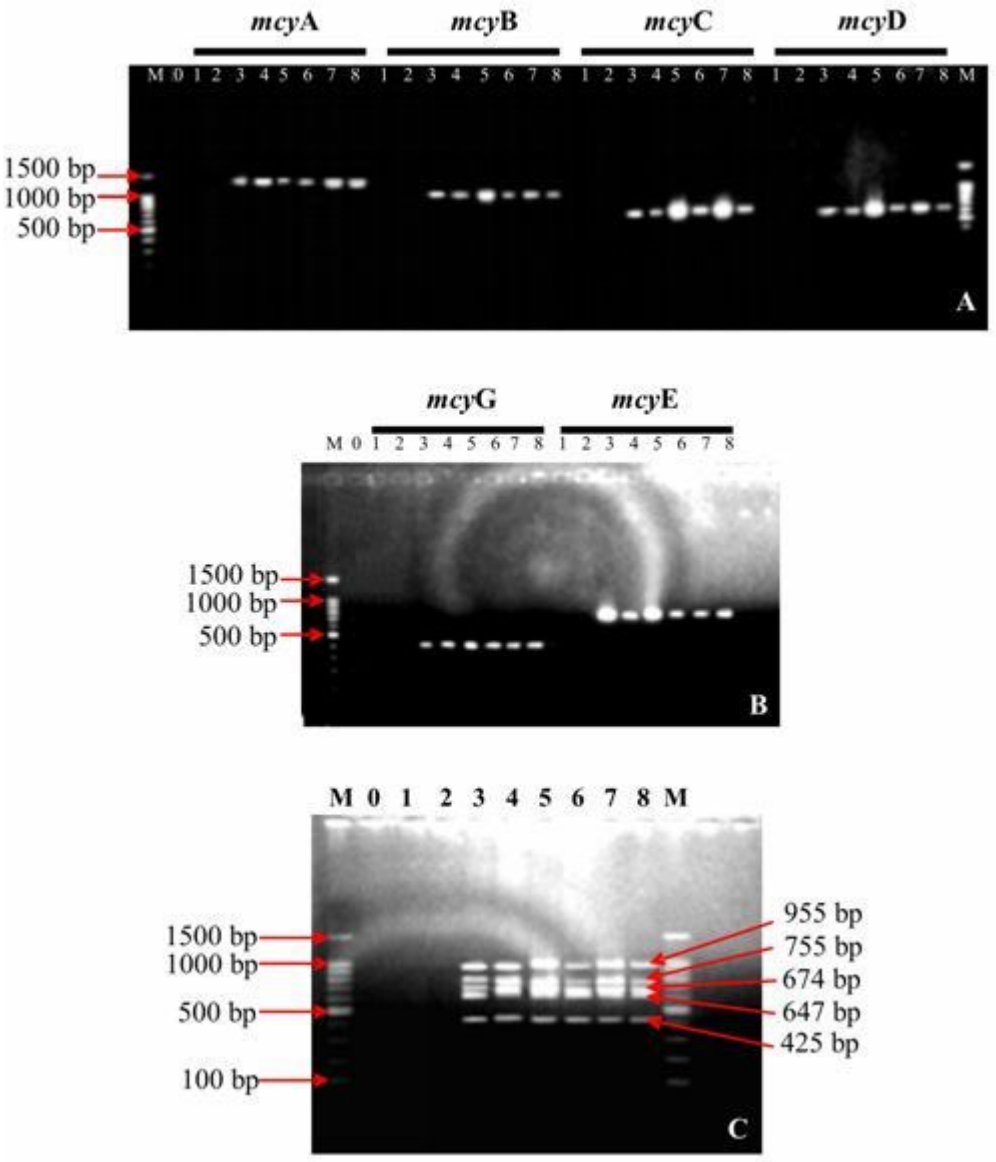

Figure 2A-C. Amplification of *mcy* genes from extracellular DNA recovered from water of various ponds. (A-B) Amplification of *mcy* genes individually (*mcyA*, *B*, *C*, *D*, *E* and *G*) by PCR, (C) Multiplex PCR for amplification of various *mcy* genes. lane 0, Negative control (without template DNA); lane1-DLW pond lacking blooms of *Microcystis*; lanes 2-8, amplification of *mcy* gene from LB; PM; AN; Kd; DK; LK; and, Gd respectively. Abbreviation of pond name is same as in Figure 1. Lane M, 100 bp DNA ladder (Promega, USA).

and *Anabaenopsis* present in blooms are different than those which are known to synthesize microcystin-LR (Kurmayer et al*.,* 2004; Kurmayer and Christiansen, 2009). That, other cyanobacteria present in bloom samples excluding *Microcystis* are not the producers of microcystin-LR was also evident from the data of microcystin content. None of the laboratory-grown unialgal cultures of these species showed the detectable level of microcystin-LR. Similar to our findings, several studies have indicated that all the strains of the reported genera (*Microcystis*, *Planktothrix*, *Anabaena*, *Nostoc* and *Anabaenopsis*) do not make microcystins, and both toxic and nontoxic strains occur in the same species (Kurmayer et al., 2004; Rantala et al., 2006; Rinta-Kanto and Wilhelm, 2006). The reasons why these species do not synthesize microcystins are not known, it is presumed that mutations within the *mcy* gene cluster might have occurred during growth under laboratory conditions

(Pearson and Neilan, 2008). Presence of inactive microcystin (*mcy*) genotypes in natural populations of *Planktothrix* has been demonstrated by Kurmayer et al. (2004). Based on above reports and results of this study, it may be concluded that *Microcystis* is the sole toxicogenic species in all the bloom samples. However, it would be appropriate to use universal primers which result in the amplification of *mcy* genes of all the known microcystin-producing species thereby avoiding the need for the isolation of strains (Rantala et al., 2006; Rinta-Kanto and Wilhelm, 2006). In this context, use of universal forward primer *mcyE*-F2 in combination with genus specific reverse primers, *mcyE*-12R*, mcyE*-R8 and *mcyE*- plaR3 has been very useful in detecting hepatotoxic *Anabaena*, *Microcystis* and *Planktothrix* species respectively. Notably, these primer sets also enabled the screening of potential microcystin-producing species in natural bloom communities (Rantala et al*.,* 2006).

In the absence of detailed molecular characterization of all the strains of *Microcystis* isolated from seven ponds, it is difficult to predict their relatedness, however, morphological features of colonies and cells suggest that all the ponds probably harbor closely related strains of *M. aeruginosa*. This presumption is also supported from the restriction analysis of *mcyA* amplicon where similarity in banding pattern was evident in blooms of all the ponds. Apparently, there are no differences in the restriction sites of *Alu*I and *Rsa*I in *mcy*A gene of *Microcystis* of different bloom samples. Additionally, analysis of *mcyA* sequence also suggested that the blooms from all the seven ponds indeed harbor *Microcystis*, and microcystin synthesis occurs by the microcystin synthetase (*mcy*) genes cluster as reported earlier (Nishizawa et al., 2000; Tillett et al*.,* 2000; Mikalsen et al., 2003; Kurmayer and Christiansen, 2009).

In the past several studies have employed molecular approaches to resolve the relationships of occurrence and toxicity of *Microcystis* and other genera (Hisbergues et al*.,* 2003; Mikalsen et al*.,* 2003; Kurmayer et al., 2004; Ouahid et al., 2005; Ouellette et al., 2006; Valério et al., 2010). Saker et al. (2007) had proposed that a multiplex PCR technique could be conveniently used to simultaneously detect the presence of microcystin-producing cyanobacteria and also *Microcystis* during one PCR. We aimed our studies with six *mcy* genes, which allowed us to show a direct relationship between the occurrence of *Microcystis* and the presence of DNA sequences corresponding to specific regions of six *mcy* genes in the blooms as well as laboratory-grown *Microcystis*. Several workers have reported that populations of *M. aeruginosa* may contain genotypes with and without one of the genes responsible for microcystin production (Wilson et al*.,* 2005). Henceforth, presence of merely one or two genes of *mcy* cluster may not be the actual indicator of microcystin-producers (Saker et al., 2007; Kurmayer and Christiansen, 2009). We could resolve this issue by selecting six genes of *mcy* cluster. Our studies differ from the previous ones mainly in two ways, (a) earlier workers used one, two or rarely three *mcy* genes for monitoring the presence of toxic *M. aeruginosa*; and (b) no attempt was made to isolate and culture cyanobacteria associated with bloom (excluding *Microcystis*) so as to test the toxicogenic property of other cyanobacteria. (Tillett et al*.,* 2001; Baker et al*.,* 2002; Kurmayer et al*.,* 2002; Pan et al*.,* 2002; Hisbergues et al*.,* 2003; Rinta-Kanto et al*.,* 2005; Valério et al., 2010).

Data of microcystin and nucleic acid content present in pond water clearly show that the release/excretion of cellular constituents indeed occurs and may remain stable in water. Similar to our findings varying levels of microcysitn in different water bodies have been reported in other studies (Rinta-Kanto et al*.,* 2005; Hotto et al*.,* 2007). Although release of microcystin may be specifically from *Microcystis* species, nucleic acids recovered undoubtedly is the sum of all the living organisms present in pond water. That there is indeed presence of *Microcystis* specific DNA in water of all the seven ponds was evident from the amplification of *mcy* genes from the DNA of water. As the environment can retain the molecular imprint of inhabiting species, our approach seems straightforward for reliable detection of organisms without observation. However, it is difficult to predict how long DNA fragments persist in water, nevertheless even traces of extracellular DNA could lead to the detection of a species such as *Microcystis* after it has disappeared from pond. It has been reported that short DNA fragments may persist in water for a long time under certain conditions, although in one report DNA from extinct vertebrates has been amplified from 10,000 year old dry cave sediments (Levy-Booth et al*.,* 2007; Ficetola et al*.,* 2008). Altogether our findings demonstrate that *Microcystis* may be readily screened by the amplification of *mcy* genes from extracellular DNA recovered from the pond water employing multiplex PCR assay. It would be desirable to estimate DNA content after the disappearance of blooms at various time intervals so as to reveal the stability of DNA as well as its suitability in PCR amplification of *mcy* genes. However, results of this investigation are encouraging and represent a step forward in the search for a simple methodology for detecting microcystin-producing *Microcystis* spp. in natural habitats.

In conclusion the multiplex PCR assay adopted by us seems useful since this can enable researchers to monitor the presence of potentially microcystin-producing *Microcystis* spp. by the amplification of *mcy* genes directly from the extracellular DNA present in the water. This approach shall avoid the problems encountered in getting enough amounts of blooms and in culturing and *in vitro* growth of *Microcystis*. It is felt that due to the sensitivity of PCR-based methods, there is the potential

to detect toxic cyanobacteria in water well in advance before the appearance of cyanobacterial bloom and detectable level of toxin concentrations.

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