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Temporal variations in microcystin-producing cells and microcystin concentrations in two fresh water ponds

Shweta Singh [a,b], Pankaj Kumar Rai [a], Rocky Chau [b], Alok Kumar Ravi [c], Brett A. Neilan [b], Ravi Kumar Asthana [a,*]

[a] Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi 221005, India
[b] School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, NSW 2052, Australia
[c] Ocular Biochemistry, All India Institute of Medical Sciences, New Delhi 110029, India

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ABSTRACT

The relationship between microcystin production, microcystin-producing cyanobacteria, including *Microcystis* spp., and various biological and physicochemical parameters in Sankuldhara and Lakshmikund, situated in the same geographical area was studied over a period of 1.5 years. Seasonal variation in cyanobacterial 16S rRNA, *Microcystis* spp. 16S rRNA, *mcyA* and *mcyB* genes were quantitatively determined by real-time PCR. *Microcystis* was the dominant microcystin producer in both study sites constituting 67% and 97% of the total microcystin-producing cyanobacteria at Sankuldhara and Lakshmikund, respectively. Microcystin concentrations were 2.19–39.60 μg/L and 15.22–128.14 μg/L at Sankuldhara and Lakshmikund, respectively, as determined by LC-MS. Principal component analysis revealed a strong positive correlation between microcystin concentration and the copy number of *mcyA* and *mcyB*, chlorophyll *a* and cyanobacterial biomass at both sites. The higher microcystin concentrations in Lakshmikund pond were attributed to the high copy number of *mcy* genes present coupled with the pond's eutrophication status, as indicated by high total algal biomass, high chlorophyll *a* content, high nutrient load and low DO. Therefore, a significant difference in microcystin concentrations, correlating with these various biological and physicochemical parameters, confirms the importance of local environmental variables in the overall regulation of microcystins production.

1. Introduction

During the past three decades frequent occurrence of toxic cyanobacterial blooms in eutrophic fresh and brackish water bodies have been reported worldwide. These toxic blooms cause significant revenue loss due to altered water quality, illness and even death of humans, animals and other eukaryotic organisms (Falconer, 1999). Globally, the most frequently occurring toxins in blooms are microcystins, produced by several genera of cyanobacteria, predominantly *Microcystis* spp. (Sivonen and Jones, 1999). Around 90 congeners of microcystins, with variable toxicity, have been reported (Neilan et al., 2012; Singh et al., 2012).

* *Corresponding author*. Tel.: +91 0542 6701101; fax: +91 0542 2366402.
E-mail address: asthana.ravi@gmail.com (R.K. Asthana).
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Changes in the composition of microcystin-producing (MC+) and non-producing (MC-) populations within a cyanobacterial bloom have been suggested to be key factors affecting microcystin levels in aquatic systems (Sivonen and Jones, 1999; Kurmayer and Kutzenberg, 2003). Previously, identification and quantification of MC+ and MC- strains were difficult due to their morphological similarity (Meissner et al., 1996). However, identification and sequencing of the *mcy* gene cluster, involved in microcystin biosynthesis (Tillett et al., 2000), has led to the development of molecular methods to screen for MC+ strains amongst cyanobacterial populations (Hisbergues et al., 2003; Kurmayer et al., 2003). These molecular methods can also detect the presence of MC+ and MC- cyanobacterial strains prior to bloom proliferation. Quantitative real-time PCR, which can detect a minimum of 11 gene copies/mL (Yoshida et al., 2007), has been used extensively for the identification and quantification of MC+ cyanobacteria worldwide (Hotto et al., 2008; Rinta-Kanto et al., 2009; Sabart et al., 2010; Al-Tebrineh et al., 2011; Conradie and Barnard., 2012); however, in India such studies are rare.

In Varanasi, India, approximately 40 freshwater ponds are used by the local population for drinking, bathing, laundry, recreation and farming. There are reports of microcystin contamination (Prakash et al., 2009; Srivastava et al., 2012) and presence of MC+ cyanobacteria in several of the Varanasi ponds (Kumar et al., 2011). *Microcystis* spp. is not the sole microcystin producer in these freshwater ponds (Kumar et al., 2011); thus, the presence of high microcystin concentrations (Prakash et al., 2009) cannot be directly linked to the presence of *Microcystis* spp. Therefore, investigations into the dynamics of microcystin production in relation to MC+ cyanobacteria and different environmental variables are imperative. This study investigated the dynamics of MC+ cyanobacteria, including *Microcystis* spp. using four independent quantitative real-time PCR assays and microcystin analysis via LC-MS. Cyanobacterial 16S rRNA and *Microcystis*-specific 16S rRNA gene PCR primers were used for quantifying total cyanobacteria and total *Microcystis* populations, respectively (Neilan et al., 1999). To study the dynamics of MC+ cyanobacteria, the *mcyA* (to quantify *Microcystis*, *Anabaena* and *Planktothrix* spp.) (Hisbergues et al., 2003) and *mcyB* (to quantify *Microcystis* spp. only) (Nonneman and Zimba, 2002) genes were targeted. In an attempt to determine the effect of local environmental variables on the dynamics of microcystin production, we studied temporal variations in MC+ and MC- cyanobacteria and the microcystin levels, and correlated the data obtained with biological and physicochemical properties of two different freshwater ponds, Sankuldhara and Lakshmikund, located in the same geographical area.

2. Materials and methods

2.1. Sampling sites and sample collection

Two freshwater ponds, Lakshmikund (25°29′69″N, 82°99′33″E) and Sankuldhara (25°30′94″N, 82°99′93″E), not connected to any water resource and mainly rain-fed, were selected for the study. Surface water samples (0–0.5 m) from four distantly located sampling points of each pond were collected in 2 L acid washed glass bottles. Sampling was conducted twice in a month between July 2010 to June 2011, and then once in a month between July 2011 to December 2011.

2.2. Strain cultivation

Microcystis aeruginosa PCC 7806 was used as reference strain in both conventional and quantitative PCR assays. The culture was maintained in BG-11 medium (Rippka et al., 1979), at 25 ± 2 °C and light intensity of 25 μmol photons/m^2/s. Cells were harvested during exponential growth phase and genomic DNA was extracted (Section 2.3) to prepare the standard curve for qPCR assay.

2.3. DNA extraction and conventional PCR

A known volume (50–100 mL) of water samples was vacuum filtered using 0.2 μm cellulose nitrate filter (Sartorius, Germany) and the retained biomass was used for DNA extraction. Filter paper, containing biomass, was crushed in liquid nitrogen and suspended in TE buffer (575 μL, pH 8.0, 10 mM Tris–HCl and 1 mM EDTA). SDS (30 μL, 10%) was added to the solution followed by proteinase-K (100 μg/mL final concentration) and mixed gently during incubation (37 °C, 1 h). Afterwards, cetyltrimethyl ammonium bromide (80 μL, 10%) was added and the mixture incubated (65 °C, 10 min). Subsequently, NaCl (100 μL, 5 M) was added. The lysate was extracted with equal volume of chloroform and isoamyl alcohol (IAA) (24:1), and aqueous phase collected after centrifugation. This was followed by extraction with equal volume of Tris saturated phenol, chloroform and IAA (25:24:1). RNAse (30 μg/mL) was added to the aqueous phase and incubated (37 °C, 30 min). Equal volume of isopropanol was added to precipitate the DNA, followed by centrifugation. The DNA pellet was washed twice with ethanol (70%) and re-suspended in 100 μL of TE buffer (pH 8.0, 10 mM Tris–HCl and 1 mM EDTA). Concentration and purity of DNA was assessed using a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies, USA).

Conventional PCR amplifications were performed by using primer sets described previously (Table 1). PCR amplification was performed in a final volume of 25 μL with 2.5 μL of 10× buffer, 2.5 μL of dNTP mixture (2.5 mM), 1.0 μL of each forward and reverse primer (10 pmol), 1 μL of template DNA and 5U of Ex-Taq DNA polymerase (Takara, Japan). Amplification was carried out with the Mastercyclerep (Eppendorf, USA). The amplification protocol was: initial denaturation at 95 °C for 5 min, followed by 40 cycles of PCR, with each cycle consisting of the first step of 15 s at 95 °C, the second step of 30 s at the specified temperature (Table 1) and the third step of 30 s at 72 °C, followed by a final elongation step of 5 min at 72 °C. Amplified PCR products were visualized on 2% agarose gels stained with ethidium bromide under UV light. PCR products were purified using QiaQuick DNA Remove kit (Qiagen) according to the manufacturer's protocol. Purified PCR products were used for sequencing to confirm the amplification of target genes. Automated DNA sequencing was performed by Prism Big Dye cycle sequencing system, using approximately 50 ng of PCR product and 3.2 pmol of one primer from the sets and analysis was done on an ABI 3730 DNA sequencer (Applied Biosystems) at the Ramaciotti Centre for Genomics, University of New South Wales, Australia.