



Ganga river water quality assessment using combined approaches: physico-chemical parameters and cyanobacterial toxicity detection with special reference to microcystins and molecular characterization of *microcystin synthetase (mcy)* genes carrying cyanobacteria

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Abstract

Water quality assessment relies mostly on physico-chemical-based characterization; however, eutrophication and climate change advocate the abundance of toxic microcystins (MCs) producing cyanobacteria as emerging bio-indicator. In the present study, a spatial-temporal analysis was carried out at ten sampling sites of Prayagraj and Varanasi during June 2017 and March 2018 to determine the Ganga River water quality using physico-chemical parameters, cyanobacteria diversity, detection of MCs producing strains and MC-LR equivalence. Coliform bacteria, COD, NO₃-N, and phosphate are the significant contaminated parameters favoring the growth of putative MCs producing cyanobacteria. National Sanitation Foundation WQI (NSFWQI) indicates water quality, either bad or medium category at sampling points. The morphological analysis confirms the occurrence of diverse cyanobacterial genera such as *Microcystis*, *Anabaena*, *Oscillatoria*, and *Phormidium*. PCR amplification affirmed the presence of toxic microcystin (*mcy*) genes in uncultured cyanobacteria at all the sampling sites. The concentration of MC-LR equivalence in water samples by protein phosphatase 1 inhibition assay (PPIA) and high-performance liquid chromatography (HPLC) methods was observed in the range of 23.4–172 ng/L and 13.2–97.5 ng/L respectively which is lower than the harmful exposure limit by World Health Organization (WHO). Ganga isolate 1 was identified as *Microcystis* based on partial 16S rDNA sequence and its toxicity was confirmed due to presence of *mcy* genes and MCs production potential. These findings suggest the presence of MCs producers as new emerging parameter to monitor water quality index and identification up to species level will be valuable for restoration strategies of river Ganga.

Keywords Cyanobacteria · *Microcystin synthase (mcy)* genes · Microcystins · HPLC · PPIA · River Ganga · WQI

Introduction

An emerging global problem is the scarcity of natural resources, especially the availability of clean water because of

population growth and economic development. River water is a vital commodity to fulfill human's daily needs and for the development of sustainable economy. The Ganges is one of India's major rivers with length of 2,525 km originating from the Gangotri glacier of western Himalayas Uttarakhand, India (Tripathi and Singal 2019). The Ganga is sacred for its purity and self-cleansing property and its holy water plays a vital role in Hindu ceremonies starting from rituals of birth, initiation of marriage and death. However, it ranked among the world's five most polluted rivers due to anthropogenic activities and climatic fluctuations (Rai 2013; Dutta et al. 2020). As per the Central Pollution Control Board (CPCB), India (CPCB 2013; UPPCB 2019), cities situated along its bank, i.e., from Kanpur to Varanasi are considered as the most polluted stream region. The intense cultivation to meet an ever-growing population's needs, encroachment of human activity and subsequent

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eutrophication due to excessive nutrient loading in water leads to the extinction of several species and vegetation (Sinha and Kannan 2014). Furthermore, the discharge of untreated sewage, industrial wastes and inflow of agricultural runoff can be considered as major determinant for pollution in the river Ganga (Dixit et al. 2017; Siddiqui and Pandey 2019). Therefore, an accurate and rational assessment of Ganga water quality using physico-biochemical and molecular approaches is an essential prerequisite for determining the extent of its usefulness in everyday life.

Due to the role of algal abundance in determining the water quality in the freshwater ecosystems, cyanobacteria have been accepted as a major bioindicator of water pollution worldwide (Mateo et al. 2015; Casero et al. 2019). The toxic strains of cyanobacteria such as *Microcystis*, *Planktothrix*, *Anabaena*, *Anabaenopsis*, *Nostoc*, *Oscillatoria*, *Aphanocapsa*, *Hapalosiphon*, *Fischerella*, *Gloeotrichia*, and *Nodularia* are the known producers of the hepatotoxic microcystins (MCs) (Kumar et al. 2020a; Wang et al. 2021). MCs are tumor promoter as they inhibit protein phosphatase type 1 and 2A in eukaryotes (Codd et al. 2005) and pose a significant health threat for drinking, irrigation water supplies and the environment at large (Turner et al. 2018; Massey et al. 2020). The biomagnification of MCs due to intake of the contaminated water by the plants and animals (fishes, water birds) will cause a potential health risk to animals and humans present at the higher trophic level (Chen et al. 2009). MCs are structurally cyclic heptapeptide synthesized by non-ribosomal pathway through multifunctional enzymes that include polypeptide synthetase and polyketide synthase modules (Neilan et al. 2013). *Microcystin synthetase (mcy)* gene cluster comprises 55 kb of DNA that includes 10 genes (*mcyA*–*J*) which are involved in the biosynthesis of MCs (Tillett et al. 2000) and essential for toxin production. The order of these ten *mcy* genes varies in cyanobacterial genera and their sequence similarities are also low (Genuário et al. 2010). More than 90 different MCs have been discovered based on degree of methylation, peptide sequence, hydroxylation and toxicity (Neilan et al. 2013). Although diverse communities of cyanobacteria are found in the river Ganga water, however, identification of toxigenic cyanobacteria that possess *mcy* genes as well as direct quantification of MCs are needed. Number of analytical techniques such as protein phosphatase inhibition assay (PPIA), enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC), mass spectrometry (MS) has been employed for the monitoring of different MCs in the contaminated water samples (Kumar et al. 2020a; Massey et al. 2020). Molecular technique such as polymerase chain reaction (PCR) based detection of *mcy* genes (*mcyA*, *mcyB*, *mcyD*, *mcyE* and *mcyG*) is rapid, cost-effective as well as facilitated the culture independent identification of toxic cyanobacteria in environmental samples (Genuario et al. 2010; Ribeiro et al. 2020). Numbers of studies were available

on the assessment of river Ganga water quality based on physico-chemical parameters, bacterial and fungal diversity and metal toxicity (Behera et al. 2020; Matta et al. 2020; Prasad et al. 2020; Dimri et al. 2020). But so far, only one report has published that monitored the pollution in river Ganga in relation to the appearance of MCs producers (two strains isolated were named *Oscillatoria* sp. RBD01 and *Leptolyngbya* sp. RBD05) along with physico-chemical parameters from the highly polluted stretch, i.e., Jajmau area of Kanpur city, Uttar Pradesh, India, for the years 2013–2015. The Ganga water was found to be positive for MCs in the studied region with concentration ≥ 2 ppb (Dixit et al. 2017).

Identification of MCs producing cyanobacteria in river Ganga has poorly monitored and under-appreciated as well as water pollution monitoring agencies do not mandate its testing for water reserve quality assessment. Hence, at regular intervals, monitoring Ganga water quality parameters, including cyanobacterial toxicants evaluation, is an indispensable requirement to decrease the water originated threats. The present study is the first regional and spatial-temporal based assessment of cyanobacteria possessing *mcy* genes and its toxin (MC-LR equivalence) concentration in Ganga water at ten different sampling sites of Prayagraj and Varanasi regions of U.P., India, accompanied by various physico-chemical and biochemical parameters. The various cyanobacterial genera present in the water samples were visualized by microscopy. The presence of potentially toxic cyanobacteria in river Ganga at study sites was monitored by PCR amplification of *mcy* (*mcyA*, *mcyB* and *mcyE*) genes. Protein phosphatase 1 (PP1) inhibition assay (PPIA) and high-performance liquid chromatography (HPLC) methods were employed to determine MCs concentration in the water samples. At the molecular level, MCs covalently bind to and inhibit the activity of enzyme PP1 which lead to hyperphosphorylation of cellular proteins and increased the carcinogenic processes or cell death (MacKintosh et al. 1990). Furthermore, isolated cyanobacterium having *mcy* genes named Ganga isolate 1 was identified as belonging to the genus *Microcystis* by morphological and molecular analysis (16S rDNA sequence). The MCs production potential of isolated *Microcystis* under culture conditions was also evaluated. Thus, this study will provide essential baseline data across the Prayagraj and Varanasi regions of UP, India to comprehend the extent, magnitude and source of MCs to predict the trophic status of river Ganga.

Materials and methods

Study area

River Ganga passes through 29 cities, 70 towns and thousands of villages. The initial oligotrophic conditions in river Ganga changed over the next 25 years to eutrophic because of

excessive nutrient loading (CPCB 2013; Dixit et al. 2017). In general, cyanobacteria have often been found to increase under eutrophic conditions. The present study was carried out at different sampling sites of Varanasi, a holy city of India, and Prayagraj, hosting the famous Kumbh Mela festival, situated on the river Ganga bank, Uttar Pradesh (U.P), India. The study area included the Gangetic stretch starting from Mehdhori Gaon Kachar (Prayagraj) to Ravidash mandir Ghat (Varanasi) with length of 130–135 km (Fig. 1) which is subjected to over-exploitation both in quantity and quality. There are various polluted drainage flows in the river Ganga from Prayagraj and Varanasi city along with more than 90 ghats with specific rituals. Indiscriminately discharged industrial effluents at Prayagraj and Varanasi regions affect physico-chemical and

microbiological water quality which provides an excellent opportunity to reproduce several algal species including toxic cyanobacteria that can be used as water quality bio-indicators. Ten different sampling locations were carefully inspected and selected at Prayagraj and Varanasi regions to analyze phytoplanktonic water samples from the river Ganga depending upon the extent of pollution (Table 1). Global positioning system (Garmin, USA) was used to determine the 10-sampling site's geographical coordinates.

Water Sampling

Water samples were collected in prewashed and sterilized PTFE bottles (Tarsons, India) from the sampling sites of

Fig. 1 Map of the river Ganga from Prayagraj to Varanasi regions of Uttar Pradesh, India, showing the 10 sampling points at the study site



Table 1 Water sampling sites of river Ganga and their coordinates at Prayagraj and Varanasi

Prayagraj			Varanasi		
Sampling sites	Latitude	Longitude	Sampling sites	Latitude	Longitude
Allahabad Sangam (S1)	25.423694	81.886056	Dashashwamegh Ghat (S6)	25.306222	83.016111
Daraganj Ghat area (S2)	25.452611	81.889111	Ramana Village Ghat (S7)	25.233583	83.028583
Mehdhori Goan kachar(S3)	25.498694	81.8475	Ravidash mandir Ghat (S8)	25.322778	83.031389
Rasoolabad Ghat (S4)	25.504028	81.856278	Samne Ghat (S9)	25.27175	83.016111
Shastri Bridge Area (S5)	25.435806	81.888389	Sheetla Ghat (S10)	25.316950	83.020670

Prayagraj and Varanasi city (Table 1) for 2 years, once during June 2017 and another in March 2018. The top layer's water samples were collected within a depth of 40–50 cm for the physico-chemical and biological parameters analysis as mentioned in Table 2. There was 10–20 L of top layer water samples concentrated to approximately 50 mL in 100-mL sterilized vials using a phytoplanktonic net of mesh size 60 μm and used for various biological analysis including algal cell density and concentration, uncultured cyanobacterial morphological evaluation, MCs producing cyanobacterial identification, MC-LR equivalence detection, isolation and characterization of MCs producing strains of cyanobacteria. A part of the concentrated water samples was fixed immediately with a Lugol's iodine solution (final concentration in sample 1%) for morphological evaluation of the cyanobacteria and algal cell concentration estimation. The collected water samples were transported to the laboratory under low-temperature conditions.

Physico-chemical and biological parameter estimation of Ganga water samples

The physico-chemical and biological parameters for water samples were estimated using standard methods for examining water and wastewater (APHA 1998). Parameters such as conductivity, pH, temperature, transparency depth (Secchi disk), dissolved oxygen (DO), total dissolved solids (TDS), and productivity were estimated onsite. The algal cell concentration was counted by a haemocytometer using microscope. For primary productivity analysis, the “Light and Dark bottle” method was followed (Benton and Werner (1972)). Water samples were collected in triplicate between 10.00 am to 12.00 noon for DO analysis of initial, light and dark bottles. Experimental bottles (light and dark) incubated underwater in the euphotic zone for two hours by suspending it in water, after which dissolved oxygen content of each bottle was estimated (APHA 1998). Primary productivity estimated as oxygen equivalent was converted into carbon equivalent by multiplying it by the factor 0.375 (Sreenivasan 1964).

Water quality index (WQI)

Water quality index of river Ganga was estimated using National Sanitation Foundation WQI (NSFWQI) method (Kamboj and Kamboj 2019) using eight different parameters (Q): NO_3-N , phosphate, TDS, pH, temperature, DO, fecal coliform and biochemical oxygen demand (BOD). The weight factor (W) of these parameters is considered as DO—0.18, fecal coliform—0.17, pH and BOD—0.12, temperature, NO_3-N and phosphate—0.11 and TDS—0.08 for river water quality. The WQI_{NSF} was calculated for each parameter by the following equation (1):

$$WQI_{NSF} = \sum_{k=1}^n W_k Q_k \tag{1}$$

where n = number of water quality parameters; Q_k = sub-index for k^{th} water quality parameter; W_i = weight factor associated with k^{th} water quality parameter.

The water quality rating scale projected was in the range of 0–100 and grading was performed as Excellent: 90–100, Good: 70–90, Medium: 50–70, Bad: 25–50, and Very bad: 0–25.

Morphological evaluation of cyanobacteria of river Ganga

Morphological evaluation of the cyanobacteria in collected phytoplanktonic water samples as well as Ganga isolates was carried under phase-contrast microscope (Nikon TE200) and photographed using a Zeiss Axioskop 60 optical light microscope equipped with an AxioVision LE 4.6 digital imaging system (Carl Zeiss, Jena, Germany).

Molecular characterization of uncultured cyanobacteria in water samples of river Ganga

Primer design for detection of cyanobacterial 16S rDNA and mcy genes

PCR based molecular characterization was done using pair of primer set 27F1 and 781R1 specific for segment of

Table 2 Physico-chemical and biological characteristics of water of the river Ganga at 10 different study sites of Prayagraj and Varanasi during 2 years (June 2017 and March 2018). Water characteristics have been compared with the permissible limit of various characteristics of drinking water

Parameters	June 2017										March 2018										Permissible limit (BIS/ICMR/CPCB/WHO)	
	Prayagraj					Varanasi					Prayagraj					Varanasi						
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10		
Physiochemical																						
Temperature (°C)	29	28	29	28	28	29	28	28	29	30	26	27	27	26	26	26	25	26	25	25	25	--
pH	7.3	7.7	7.5	7.6	7.8	7.6	7.7	7.5	7.6	7.7	7.8	7.9	7.5	7.6	7.6	7.5	7.5	7.7	7.6	7.8	6.5-8.5	
Conductivity (mho/cm)	304	293	308	402	374	309	352	293	386	372	273	245	294	304	234	209	191	284	309	375	300 mho/cm	
TSS(mg/L)	89	142	118	123	80	102	62	135	124	96	96	167	108	156	78	93	102	132	95	109	---	
TDS(mg/L)	257	289	343	320	261	309	285	321	298	310	221	209	275	298	248	294	283	283	270	227	500/500/-/500 mg/L	
Hardness(mg/L)	284	301	290	212	289	210	194	298	217	313	374	290	294	192	285	192	176	289	288	276	300/600/600/500 mg/L	
Alkalinity (mg/L)	163	128	183	165	162	145	132	193	141	197	114	102	148	134	128	165	122	184	124	184	120 mg/L	
Iron(mg/L)	0.152	0.161	0.180	0.157	0.169	0.172	0.152	0.162	0.218	0.190	0.132	0.138	0.130	0.152	0.143	0.173	0.150	0.148	0.215	0.179	0.3/1.0/1.0/0.1 mg/L	
NO ₃ -N(mg/L)	3.78	3.72	3.58	2.94	4.56	4.32	3.91	2.84	4.32	4.92	2.74	3.12	2.94	2.82	2.63	3.25	2.52	2.74	2.83	2.56	20/20/-/45 mg/L	
COD(mg/L)	41.2	32.8	48.4	50.7	43.9	44.2	54.3	50.2	43.6	41.4	37.2	33.3	36.2	39.4	40.5	42	31.2	42.5	30.7	42.5	---	
Phosphate (mg/L)	1.32	1.45	1.78	1.21	1.31	1.64	1.82	1.32	1.29	1.49	1.04	1.14	0.98	1.04	1.44	1.05	1.68	0.92	0.99	1.17	---	
Sulphate (mg/L)	46.5	40.0	49.0	45.2	41.0	52.0	37.4	45.2	40.4	50.2	36.2	32.3	39.5	37.2	39.2	46	42.1	36.5	37.2	35.0	150/400/400/-/ mg/L	
Chloride (mg/L)	102	65	71	82	89	101	104	88	89	94	74	69	72	81	82	83	65	91	70	92	250/1000/1000/200 mg/L	
Dissolved Oxygen (mg/L)	7.1	7.1	6.8	6.7	7.4	7.6	7.8	6.9	8.0	6.8	7.4	7.2	7.3	7.2	7.6	7.5	8.1	7.6	8.2	7.3	>4.0 mg/L	
BOD _{5day} (mg/L)	4.8	3.9	4.1	4.9	4.7	4.5	4.3	4.4	4.2	4.1	3.7	3.9	3.8	4.0	3.9	3.2	3.6	3.3	3.5	3.7	3.0/-/4/4 mg/L	
Biological																						
Secchi transparency depth (m)	0.50	0.45	0.37	0.35	0.48	0.42	0.50	0.39	0.44	0.41	0.54	0.62	0.44	0.46	0.52	0.42	0.60	0.48	0.52	0.51	---	
Algal cell conc. (cells/mL) x 10 ³	8.1	9.0	9.3	10.2	10.7	10.1	9.7	8.7	8.3	9.2	7.3	7.1	6.4	6.8	5.7	8.1	6.3	4.1	3.1	9.2	---	
Algal cell density (mg/L)	23.8	25.0	20.6	25.2	22.1	26.3	24.3	28.4	23.7	28.4	17.6	16.1	17.9	19.2	15.2	18.0	16.3	13.3	12.3	17.8	---	
Total coliform (MPN/100mL) x10 ³	7.6	8.9	10.3	11.3	9.3	10.3	8.2	9.5	11.3	11.2	6.2	5.3	7.3	6.9	8.4	6.9	7.2	6.5	6.3	6.1	--/4/4/No relaxation	
Fecal Coliform (MPN/100mL) x10 ³	4.0	5.3	6.1	7.2	4.8	7.1	4.9	5.2	8.1	7.2	3.1	3.5	4.0	4.4	3.2	3.8	3.1	3.3	4.1	4.4	--/4/4/No relaxation	
Chlorophyll a (mg/L) x10 ⁻²	1.40	1.64	1.74	2.17	1.30	1.52	1.62	1.80	2.21	1.49	0.90	1.02	1.10	0.86	1.10	1.14	0.92	0.92	1.21	0.84	---	

Table 2 (continued)

Parameters	June 2017					March 2018					Permissible limit (BIS/ICMR/CPCB/WHO)										
	Prayagraj					Varanasi															
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Productivity GP (mgO ₂ /m ² /24h)	30.8	20.4	20.7	19.3	22.9	19.8	20.6	22.7	22.9	27.4	20.9	28.2	30.2	28.4	26.8	25.6	30.2	29.3	20.5	18.5	---
NPP	12.3	11.4	12.9	9.5	10.2	13.0	10.3	11.5	11.9	12.7	10.4	14.6	11.2	13.2	14.2	12.2	16.9	11.7	10.2	9.7	---

Sampling sites-PRAYAGRAJ: (S1) Allahabad Sangam,(S2) Daraganj Ghat area, (S3) Mehdhori Gaon Kachar,(S4) Rasoolabad Ghat, (S5) Shastri Bridge area;VARANASI: (S6) Dashaswanegh Ghat, (S7) Ramana Village (S8) Ravidash mandir Ghat,(S9) Sarame Ghat, (S10) Sheetla Ghat; GP gross productivity; NPP net primary productivity. Each experiment was performed in triplicates and presented in the mean value. S.D. was observed less than 5%

cyanobacterial 16S rDNA (Jungblut et al. 2005). The specific *mcyA*-Cd primer pair was designed from a highly conserved region coding for the condensation domain of the *mcyA* gene that would allow the specific amplification from *Microcystis*, *Planktothrix* strains as well as other MC producing cyanobacterial genera (Hisbergues et al. 2003). For *mcyB* gene, the oligonucleotide primer set used in this study was designed by Mikalsen et al. (2003). To amplify the glutamate-activating adenylation domain of *mcyE* gene, primer pair was designed from general *mcy* gene E forward primer (*mcyE*-F2) and reverse primer (*mcyE*-R4), the genus-specific reverse primer for *Microcystis* (*MicmcyE*-R8), *Planktothrix* (*PLAmcyE_R3*) and *Anabaena* (*AnamcyE*-R12) (Rantala et al. 2004; Gobler et al. 2007). The specificity of each designed primer for *mcy* genes was verified by running BLASTn in the NCBI databases.

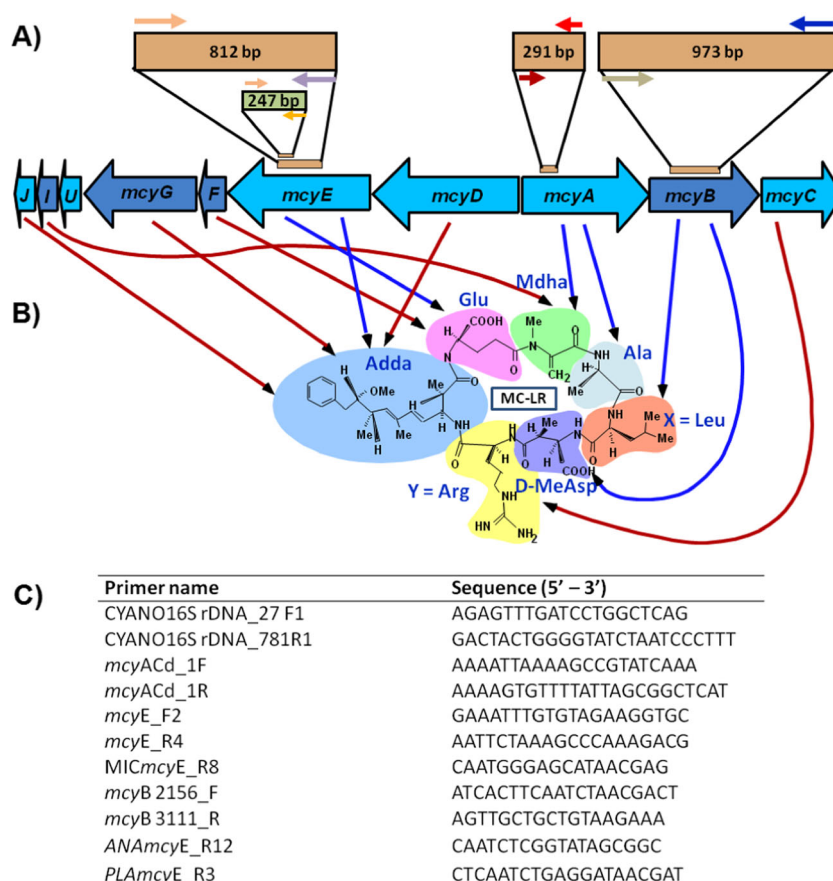
Genomic DNA extraction and PCR amplification

The presence of cyanobacteria having toxic *mcy* genes at ten sampling sites was investigated by PCR method using set of primers and strategies mentioned in Fig. 2. Genomic DNA extraction from the uncultured cells of phytoplanktonic Ganga water samples was performed according to the protocol of Srivastava et al. (2007). The concentration and purity of genomic DNA was checked by measuring absorbance at 260 nm and A₂₆₀/A₂₈₀ ratio in NanoVue Plus spectrophotometer (GE Healthcare Life Sciences) and was immediately kept at -20 °C for future purpose.

To PCR amplify the regions of genomic DNA targeting portion of the cyanobacterial 16S rRNA and *mcy* genes, seven primer pairs were used (Fig. 2). Each PCR reaction consists of 20 µl that includes 20 ng/µl of template DNA, 1 x PCR buffer (GeNei Lab, India), 0.2 mM each dNTPs (GeNei Lab, India), 5 pM of each primer and 0.05 U of Taq polymerase (GeNei Lab, India). The reactions were performed in Mini Thermal Cycler (BioRad) programmed for 35 cycles. The initial denaturation step was at 94 °C for 5 min. followed by 34 cycles of 94 °C (45 s), 50 °C (60 sec), and then 72 °C (90 s). An additional cycle of final extension at 72 °C (10 min) was used. The PCR products were electrophoresed in 1.5 % agarose gels stained with ethidium bromide (1 µg/ml) using 1x TAE electrophoresis buffer (pH 8.0). The sizes of amplified products were compared with the 100 bp DNA ladder (BR Biochem Life Sciences Pvt. Ltd.) to determine the size of amplified products. The gel was photographed in the G:BOX gel documentation system (Syngene, USA).

For further confirmation of microcystin synthase (*mcyA*, *mcyB*, and *mcyE*) genes in uncultured cells, the PCR amplified products of the desired length were purified using QIAquick gel extraction kit (Qiagen). Blunt end DNA fragments (insert) generated were cloned in the pJET1.2/blunt cloning vector by CloneJET PCR Cloning kit (Thermo

Fig. 2 Primers and strategy used for the detection of microcystin synthetase (*mcy*) genes possessing cyanobacteria in the phytoplanktonic water samples of river Ganga at 10 sampling points of Prayagraj to Varanasi. A) Schematic arrangement of the 10 genes *mcyA*-*J* in MC operon. B) Structure of cyanobacterial heptapeptide heptatoxins MC-LR [cyclo-(D-Ala-X-D-MeAsp-Y-Adda-D-Glu-Mdha)]; X, L=leucine; Y, L=arginine; Me=CH₃; D-MeAsp=D-erythro-β-methylaspartic acid; Adda=(2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; Mdha=N-methyldehydroalanine (dha=dehydroalanine). C) Details of primers used in the study



scientific). After ligation, the recombinant plasmids were transformed into *E. coli* strain DH5α (Novagen) and confirmed using colony PCR and subsequently by plasmid PCR. Recombinant plasmids harbouring *mcyA*, *mcyB* and generic *mcyE* genes were sequenced from GeNei Laboratories (India) for further phylogenetic studies. Each of the three *mcy* (*mcyA*, *mcyB*, and generic *mcyE*) partial gene sequences obtained from this study were compared and analyzed using the reference sequences retrieved by BLASTn program available at GenBank and were aligned using ClustalW multiple sequence alignment. Phylogenetic tree analysis was performed using the neighbour-joining method based on the Kimura 2-parameter distance model. The tree robustness was estimated by bootstrap analysis based on 1000 resampling. The MEGA 6.0 program package was used for all the analyses (Tamura et al. 2013).

MC-LR equivalence detection

For MC-LR equivalence detection, the Ganga water samples collected in March 2018 from ten different study sites were frozen immediately or within one hour in lyophilized flasks of minimum 100 ml volume and kept for lyophilization (−70°C). The lyophilized residues were extracted with 70% methanol as described by Fastner et al. (1998). The supernatant of

centrifuged samples was analyzed for MC-LR equivalence by the PPIA and HPLC methods.

PPIA for MC-LR equivalence detection

Pure MC-LR and protein phosphatase 1 (PP1) enzymes were obtained from ENZO Life Science (USA). HPLC grade methanol, dimethyl sulfoxide (DMSO) and p-nitrophenyl phosphate (*p*NPP) were procured from Merck and HiMedia, respectively. The stock solution (1 g/L) of MC-LR was used for preparing the MC-LR working solution (1 mg/L) using methanol for the standard inhibition assay. The standard solutions of pure MC-LR was prepared from working solution in the concentration 0.01, 0.1, 1, 5, 10, 100, 200, 300, 500, and 1000 μg/L respectively in clean glass vials and used for dose-response inhibitory activity of MC-LR on PP1 using *p*-NPP substrate. The stock solution of purified PP1 enzyme was prepared by dissolving in DMSO (1 mg/mL). The working concentration of PP1 enzyme (1 μg/mL) was prepared in buffer [50 mM Tris-HCl, 0.5 g/L BSA, 2mM MnCl₂, 0.1% β-mercaptoethanol, 1 mM Na₂EDTA, pH 7.4] to. The *p*NPP of 15 mM concentration was prepared in buffer containing 50 mM Tris-HCl, 0.2 mM MnCl₂, 20 mM MgCl₂, pH 8.1.

The standard curve assay was carried out by adding 10 μL each of pure MC-LR standard solution and PP1 enzyme

working solution in a 96-well polystyrene microtitre plate. After pre-incubation for 1 min, 180 μL of the substrate was added in a PP1 enzyme solution. The *p*-nitrophenol (pNP) production rate was estimated at 3 min interval for 21 min at 410 nm on a microtiter plate reader (Synergy, BioTek) at 28 °C. All enzymatic assays were performed in triplicate.

The assay for the test solution of MC was performed in a similar way as standard curve assay. Blank was prepared using a 10- μL standard solution of pure MC-LR, 10 μL enzyme dilution buffer and 180 μL of *p*-NPP substrate. The positive control was investigated on the PP1 activity using a 10 μL PP1 enzyme solution, 10 μL methanol and 180 μL of *p*-NPP substrate. For PPIA, the absorbance and PP1 enzyme kinetics profiles were obtained at 405 nm using the microtiter plate reader (Synergy, BioTek). The enzymatic reaction was performed at 37 °C for 21 min (3 min of an interval).

HPLC method

HPLC analytical column C18, ODS (25 \times 0.46 cm and 5 μm particle size) was used for MC-LR equivalence detection and the volume of samples injected was 100 μL . UV array detector attached to the HPLC system (Waters System, USA) was used for detection at 238 nm. The column temperature was maintained at 30 °C by column oven. Two mobile phases (acetonitrile and HPLC water) were applied with TFA (0.05% v/v) for detection. The applied analytical procedure was gradient run as follows: 0 min—20% A and 80% B, 8 min—60% A and 40% B, 15 min—20% A and 80% B, 25 min—20% A and 80% B. The flow rate was kept at 1.0 mL/min. The standard calibration curve was used for the determination of the unknown concentration of MC-LR equivalence in samples.

Isolation and characterization of MC producing cyanobacteria from river Ganga

Isolation of cyanobacteria possessing *mcy* genes was attempted from river Ganga water samples using standard isolation protocol in BG-11 agar plate and liquid media. Phytoplanktonic water samples (500 μL) were streaked on BG-11 agar (1.5% w/v) plates and incubated at 27 \pm 1 °C under the fluorescent light tubes emitting 72 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ light intensity with a photoperiod of 14:10 h. The cyanobacterial inoculum was transferred to the new BG-11 agar plates. The process was continued until single colonies of cyanobacterial strains were obtained. The purified isolates of cyanobacterial strain were re-cultured in BG-11 liquid media at similar conditions for 4–8 weeks. Preliminary identification of the isolated strains was based on morphological characters as observed under microscope which was further evaluated for the presence of *mcy* genes using PCR approach. The Ganga isolate possessing *mcy* genes was further estimated

for its genetic identity and MC producing potential. For genetic identification of the Ganga isolate possessing *mcy* gene, partial 16S rDNA sequence obtained was aligned and analyzed with reference sequences of different closely related cyanobacterial species retrieved from the GenBank database.

Statistical analysis

The physico-chemical and biological parameters data of water samples were presented as mean values with standard deviation. For WQI analysis, descriptive statistics were applied. Box-whisker plot was used to describe the spatial-temporal variations of the observed water quality parameters of river Ganga.

Results and discussion

River Ganga is the primary water source for wider communities in North India. Prayagraj, and Varanasi cities are densely populated areas with intense agricultural activities therefore, the Gangetic water stretches in these regions are characterized by high nutrient inputs from the catchment and categorized as eutrophic (Kumar et al. 2020b). The statistics about the river Ganga surface water quality are crucial for various usages and preserving its aquatic life. So far, information about the distribution of MCs producing cyanobacteria is uncertain in Ganga water, which is essential to estimate the health risks associated with the cyanobacterial toxins. Taking recourse to above, the present study was executed to assess the river Ganga's surface water quality at ten different sampling sites in Prayagraj and Varanasi using various physico-chemical and biological characteristics. The monitoring approach for Ganga water further involves the detection of putative MCs producing cyanobacteria and its toxin MC-LR equivalence. Table 1 describes river Ganga's water sampling points and their geographical coordinates (latitude, longitude) at study sites.

Physico-chemical and biological parameters of river Ganga water at Prayagraj and Varanasi

The physico-chemical and biological parameters of river Ganga water at various sites of Prayagraj (sites: S1–S5) and Varanasi (sites: S6–S10) with their mean values were estimated and shown in Table 2. A total of 15 physico-chemical and 7 biological parameters were investigated for 2 years, i.e., June 2017 and March 2018, respectively. The study suggested both spatial and yearly variations in the physico-chemical and biological parameters.

Physico-chemical parameters analysis

It is clearly depicted that (Table 2) the pH level ranges between 7.5 and 7.9 at all sampling sites during the study time and follows the standards provided by Bureau of Indian Standards (BIS), Indian Council of Medical Research (ICMR), CPCB and World Health Organization (WHO). The slight difference in pH value was due to the low annual disparity in free CO₂ in the sampling regions for the 2 years of study. Singh et al. (2016), Siddiqui and Pandey (2019) and Pandey et al. (2015) reported studies of physico-chemical parameters of the Ganga river and observed that the range of pH was 7.1–7.8, 7.6–7.8, and 8.2–8.45, respectively at different sites of Varanasi which is in accordance with the present study. However, Pandey et al. (2014) shown that the Prayagraj region's pH range was 8.1–8.4. Similar results were reported by Dixit et al. (2017), Kumar et al. (2020b) and Satya and Narayan (2018) for pH values that ranged from 7.4–8.1, 7.6–8.8, and 6.0–8.0 in river Ganga at nearby cities Kanpur and Patna.

The dissolved solids including chloride, calcium, and magnesium salts in the water samples were responsible for the conductivity level. According to BIS, CPCB, ICMR and WHO, the maximum allowable conductivity level is 300 mho/cm in the water. The measured conductivity values for water samples at sampling sites in the river Ganga varies between 191–386 mho/cm (Table 2). The average conductivity was more (336.2 and 342.4 mho/cm) in June 2017 and slightly lowers (273 and 274 mho/cm) in March 2018 than the permissible limit in both the cities. This change may be due to the seasonal change or Ganga clean drive by government initiatives. Although there is no direct impact of water conductivity on human health but its estimation is required to evaluate the mineralization rate of present mineral salts and determination of the number of chemical reagents implied to treat the water (Kavcar et al. 2009). Higher conductivity leads to the lowering of drinking water's aesthetic value, eliminating habitat-forming plant and corrosion of the metal surface of industrial equipments such as boiler (Rahmanian et al. 2015).

The maximum threshold limit of total suspended solids (TSS) in drinking water and the freshwater stream is not mentioned by any water regulatory bodies (Kumar and Puri, 2012). However, norms used in the guideline are stated and based on indirect effects on human health due to particulate matter. Water regulatory authority of some countries such as National Water Quality Standard (NWQS) Malaysia, provided the maximum threshold limit of TSS 150 mg/L for rivers which support aquatic life (Al-Badaïi et al. 2013). The TSS values in water samples of river Ganga at study sites were observed in the range of 62–167 mg/L (Table 2). The average TSS value for June 2017 was observed 107.1 mg/L, whereas the average TSS value during March 2018 was found 113.6 mg/L. This change in the TSS value is not substantial in two

different years of study. Pandey et al. (2015) reported the TSS value range 60–117 mg/L in the Ganga water samples at different sites of Varanasi Ghats during 2012 and 2013.

The total dissolved solids (TDS) values in the Prayagraj region at different sites range from 209 to 343 mg/L. The highest value obtained was 343 mg/L recorded at site S3 (*Mehdhori Gaon Khachar*) during June 2017, and the lowest value obtained was 209 mg/L at site S2 (*Daraganj ghat*) during March 2018 (Table 2). Additionally, the TDS concentrations in the sampling sites of Varanasi ranged from 227 to 321 mg/L during 2 years of study. The lowest concentration was 227 mg/L recorded at site S10 (*Sheetala Ghat*) and the highest concentration was 321 mg/L at site 8. In the present study, the TDS concentration observed was less than reported for the Prayagraj and Varanasi areas in the range of 284–707 mg/L and 454–703 mg/L respectively (CPCB 2013). It was noticed that upstream and downstream sites of both cities have TDS values with no significant difference because anthropogenic and land use activities were expanded more in both ends. Moreover, TDS values are within the permissible level (500 mg/L) set by BIS, CPCB and WHO (Kumar and Puri 2012). Pandey et al. (2015) also reported the concentration range of TDS in river Ganga at Varanasi and Prayagraj, the values obtained were 266–344 mg/L and 185–271 mg/L, respectively.

The hardness of water is not a pollution indicator but signifies water quality primarily caused by the presence of Ca²⁺ and Mg²⁺, HCO₃⁻, SO₄²⁻, Cl⁻, and NO₃⁻ in water. The hardness of water has no known adverse influences on health (WHO 2011). However, some evidence has pointed out its impact on human health, mainly stone formation in kidney and heart diseases, if the water with hardness of 150–300 mg/L or above can be consumed at a rate of 2 l per day (Mitra et al. 2018). Water has been classified as soft, moderate, hard and very hard based on hardness value >60, 60–120, 120–180, and <180 mg/L of CaCO₃, respectively (McGowan 2000). The average value of water hardness recorded was 275.2 mg/L and 246.4 mg/L in June 2017 for Prayagraj and Varanasi sampling sites respectively. The hardness values of water samples during March 2018 were more or less similar (287.0 and 244.2 mg/L). Thus, the negligible variation was observed in the water hardness for 2 years (i.e., June, 2017 and March, 2018) at study sites. The water hardness at study regions observed was within the range of permissible limit (300 mg/L) recommended by BIS. These finding was in agreement with Kumar et al. (2020b), who mentioned similar pattern of the hardness of Ganga water at the middle Ganga plains in different seasons. Alkalinity in water is due to the presence of carbonates, bicarbonates and hydroxides. Alkalinity recorded the highest average value of 161.6 mg/L during the June 2017. The range of alkalinity was observed between 114 to 197 mg/L during 2 years of study at different sites (Table 2). The average value of alkalinity was 160.2 mg/L in June 2017

and 125.2 mg/L in March 2018 at Prayagraj regions. The average level of alkalinity was observed 161.2 mg/L in June 2017 and 155.8 mg/L in March 2018 at Varanasi regions. The value of alkalinity in the study site was found at the upper side of the permissible limit (120 mg/L) as per BIS records. Pandey et al. (2015) also demonstrated a higher range of total alkalinity (230–279 mg/L) in river Ganga at Varanasi.

Increased nutrients (nitrates and phosphates) are among the primary reasons for low water quality in the freshwater stream (Davie 2003). Agriculture and urban runoff include fertilizer, domestic, sewage and industrial wastewater discharges are major routes of entry into water bodies in terms of nutrient pollution (Dubey et al. 2012). Nitrate and phosphate concentrations in freshwater can cause oxygen depletion that resultant in the deterioration of aquatic life. Consumption of high concentrations of nitrate and phosphate contaminated water causes the blue baby syndrome, muscle damage, breathing problems, and kidney failure (Davie 2003). The maximum permissible limit of NO_3^- -N level is in the range of 20–45 mg/L set by BIS, ICMR and WHO (Table 2). In the present study, the NO_3^- -N concentration observed was ranged between 2.52 and 4.92 mg/L. The decrease in overall level in the NO_3^- -N due to temporal effect was observed ~28% in Ganga water. The concentration of nitrates observed in Ganga water was below the level as prescribed by water regulatory authorities. In this study of 2 years, PO_4^{3-} -P concentration was observed in the range of 0.92–1.82 mg/L at Prayagraj and Varanasi's sampling sites. The average concentration of phosphate content was 1.46 mg/L and 1.15 mg/L during June 2017 and March 2018, respectively. The trend of phosphate concentration in the particular region was more or less similar and not much effect of time was observed.

Chloride is present naturally in all water types; however, its major contribution is the runoff of inorganic fertilizers from agricultural land and sewage discharge (Lkr et al. 2020). The chloride concentration variation was 65 mg/L (at site 2 and 7) to 104 mg/L (at site 7) in two different times for 2 years (June 2017 and March 2018). The variation was recorded ranging between 65 to 104 mg/L and 65 to 92 mg/L during June 2017 and March 2018, respectively. In comparison, Singh (2010) and Pandey et al. (2015) were recorded the spatial-temporal variation of chloride concentration in Ganga water in the range of 8.2–81.5 mg/L and 21.4–94.7 mg/L at the Varanasi region. Pandey et al. (2014) reported the variation of chloride concentration in water samples at Prayagraj, ranging between 8.2 and 21.4 mg/L. According to Hem (1985), the major sources of sulfate in the freshwater stream are rocks weathering and human activities such as mining, water discharge and fossil fuel combustion. The sulfate values varied from 32.3 (site S2) to 49.0 mg/L (site S3) at Prayagraj and 35.0 (site S10) to 52.0 mg/L (site S6) at Varanasi during sampling periods. A similar range of results (37.9–54.2 mg/L and 16–36 mg/L) were also obtained in Ganga water samples

at Varanasi by Pandey et al. (2015) and Singh et al. (2016), correspondingly. The chloride and sulfate concentration in the Ganga water samples observed were within the permissible limit suggested by BIS, ICMR and CPCB. The iron content in the sampling sites ranges from 0.13 to 0.218 mg/L. Iron content was recorded highest at site S9 (*Samane Ghat*, Varanasi) during 2 years of study. The iron level in studied samples fell within the acceptable limit (0.3–1.0 mg/L) of BIS, ICMR and CPCB, except the WHO's water specification (0.1 mg/L).

Chemical oxygen demand (COD), biochemical oxygen demand (BOD), and dissolved oxygen (DO) are important parameters for water quality evaluation. They reflect the physical and biological processes prevailing in the water that indicates the degree of pollution in water bodies. During the study, the spatial-temporal variations in COD, BOD and DO recorded were 30.7–54.3, 3.2–4.9, and 6.7–8.2 mg/L at the ten selected sites. The slight difference (0.3 mg/L) in average DO concentration may be mainly due to the water temperature difference of about 4°C. The dissolved oxygen's desirable limit is more than 4 mg/L as per BIS and CPCB standards. Pandey et al. (2015) observed that the COD, BOD and DO values range between 53.5–79.6, 37.4–58.7, and 3.7–5.8 mg/L, respectively, in the Ganga river of Varanasi region. CPCB (2013) also recorded the range of COD, BOD and DO concentration 46.2–156.2, 3.7–9.6, and 7.0–7.8 mg/L correspondingly at Varanasi region and 17.3–192, 2.6–5.6, and 6.0–9.8 mg/L respectively at Prayagraj region. The DO level recorded at all the sites is sufficient for the planktons to survive and perform various physiological water activities (Al-Badaii et al. 2013).

Biological parameters analysis

Transparency is a measure of the water's clarity and is essential for the survival of aquatic plants that require sunlight for photosynthesis. Transparency depth values can be influenced significantly by numerous factors such as day time at which measurement has recorded clearness of the sky (cloudy, partly cloudy or sunny) and suspended solids in water, including plankton (Verma and Saksena 2010; Sarkar et al. 2019). The value of transparency depth recorded was from 0.35 to 0.62 m for 2 years in the present study. Changes in values at the study sites are possibly due to the differences in bottom sediments and temperature, dissolved organic matter or entry of municipal and industrial waste material. The higher value of transparency depth noted was during March 2018 (0.51 m) than June 2017 (0.43 m). A similar kind of variation in transparency depth was demonstrated by Matta et al. (2017).

During this study, the spatial-temporal variation in algal cell concentration and algal cell density observed were in the range of 3.1×10^3 – 10.7×10^3 cells/mL and 12.5–28.4 mg/L respectively. The chlorophyll-a (Chl-a) concentration range was found 0.84×10^{-2} – 2.21×10^{-2} mg/L. The variation in three

biological parameters (Chl-a, algal cell concentration and algal cell density) are closely related (Table 2). Eutrophication and global warming are the main reasons to promote the excessive growth of algae globally in freshwater bodies (Davis et al. 2009; Sarkar et al. 2019). The toxic cyanobacterial occurrence causes many problems for instance, bad odour, low esthetic value, water quality deterioration and oxygen depletion in water which can impair tourism, transportation and ecosystem health (Son et al. 2015).

The increase in nutrient load in water bodies leads to high productivity that modifies the structure of water communities. Productivity measurement, algal and bacterial concentrations are important environmental change indicators that include acidification, climatic change and eutrophication (Dam et al. 1994). However, these biological parameters varied by numerous factors such as nutrients, temperature and organic matters. The gross productivity (GP) and net primary productivity (NPP) at different Prayagraj and Varanasi sites recorded were between 18.5–30.8 mg C/m²/h and 9.5–16.9 mg C/m²/h for 2 years of study when the average sunlight per day was 10 h. The increase in NPP to GP value approximately 6.5–11.9 % was found in March 2018, which could be due to higher water transparency in March 2018. Limited studies are available concerning the assessment of productivity and biological indicators and their effect on Ganga water quality (Siddiqui and Pandey 2019; Pandey and Yadav 2015). Pandey and Yadav (2015) reported that the GP at different sites in the Ganga river of Varanasi region ranged from ~2–8.5 mg C/m²/h, which are in agreement with our results. Productivity variables such as GP, Chl-a, and algal biomass showed synchrony with the concentration of nutrients and indicates that the river Ganga polluted at Varanasi and Prayagraj regions.

Fecal coliform and total coliform concentration in both regions observed were higher than the criteria at most locations, while the highest value always observed were at Varanasi (site-S9, Samne ghat; 11300 MPN/100 mL). CPCB (2013) reported fecal coliform and total coliform level 8000–46000 and 13000–70000 MPN/100 mL respectively in the 6-year Ganga survey (2006–2011) for the Varanasi region. These biological parameters observed were also in the range of 3000–5000 and 7000–14000 MPN/100 mL respectively in the Prayagraj region.

Spatial-temporal and Water Quality Index (WQI) analysis

The descriptive statistics for spatial-temporal variation of water quality parameters at ten sampling sites of Prayagraj and Varanasi city studied for 2 years are presented by the box-whisker plot in Fig. 3 (a) and (b). Box and whisker plots represent the full spatio-temporal dynamics of 2-year studied physical, nutrient, chemical, ionic and biological parameters

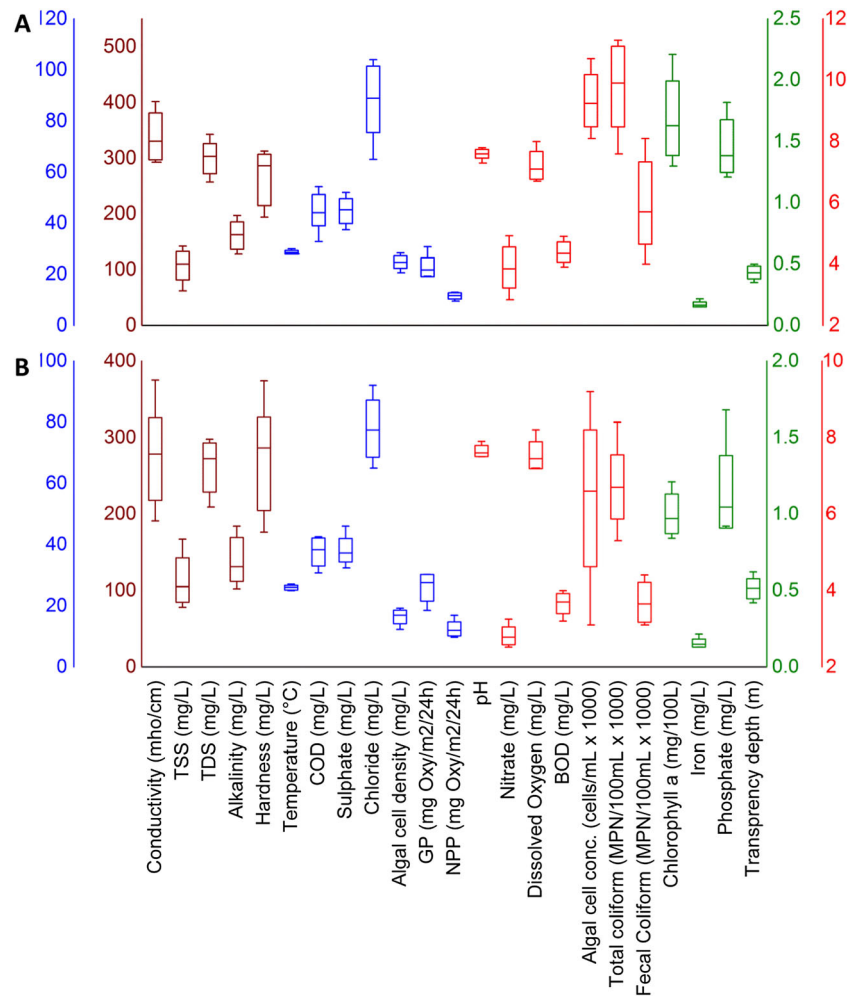
investigated for the river Ganga water samples. For most parameters, the higher standard deviation indicates temporal and spatial variations likely caused by polluting sources and/or climatic factors.

The WQI approach applied to assess river Ganga's water quality intending to provide a valid and straightforward method for expressing several parameters rapidly and conveniently. Eight parameters employed by WQI can indicate the water quality at various sampling points of Prayagraj and Varanasi regions. The average WQI score obtained was 46.31 and 50.66 for two different times, June 2017 and March 2018, respectively (Table 3). It was apparent that the water samples of study sites were falls under medium and bad category for drinking or bathing. WQI value observed was mainly due to the high concentration of fecal coliform in the water samples. Based on WQI, water qualities identified at sampling points in the river may indicate that it can be suitable for transportation, irrigation and water supply purposes. Comprehensively, comparable research was conducted in many countries such as surface water of Amazonia Rivers, Brazil (Medeiros et al. 2017), Sarayduzu Dam Lake, Turkey (Kükrer and Mutlu 2019), Kafr El-Sheikh Governorate, Egypt (Jahin et al. 2020), and the Cau river, Vietnam (Son et al. 2020). WQI analysis showed that Ganga water in the sampling regions approaches medium/bad quality conditions but increased human activity warns us about future consequences. Various WQI studies has been recently performed in the various stretches of river Ganga (Kumar et al. 2021; Dimri et al. 2020; Kumar et al. 2020b).

Morphological evaluation of cyanobacteria of river Ganga

Morphological observation of phytoplanktonic water samples by microscopy revealed the diversity of cyanobacterial genera in river Ganga at study sites (Supplementary Fig. 1). It was observed that colonial and buoyant cyanobacteria were abundant preferably the genus *Microcystis*. Diverse cyanobacterial colonies showed variations in individual cell sizes, cell arrangement, colony morphology and mucilage characteristics. Besides *Microcystis*, many other species belonging to the genera *Leptolyngbya*, *Planktothrix*, *Anabaena*, *Gleocapsa*, *Phormidium*, *Hormogonia*, *Oscillatoria*, *Aphanizomenon*, *Aphanocapsa*, and *Scyneococcus* were also observed (Supplementary Fig. 1). Similarly, Rishi and Awasthi (2015) recorded the most dominating genera in river Ganga at Kanpur, U.P, India were *Anabaena*, *Microcystis*, *Cylindrospermum*, *Phormidium* *Aphanizomenon*, *Chroococcus*, *Lyngbya*, *Nostoc*, *Nodularia*, *Spirulina*, and *Oscillatoria*. Other studies have also demonstrated the widespread occurrence of *Oscillatoria*, *Leptolyngbya*, *Scyneococcus*, *Anabaena* in river Ganga (Shukla et al.

Fig. 3 Box and whisker plot represents the spatial variation in various physico-chemical and biological parameters of Ganga water at 10 different study sites of Prayagraj and Varanasi during **A)** June 2017 and **B)** March 2018. GP- Gross Productivity; NPP- Net Primary Productivity



2015; Dixit et al. 2017; Sarkar et al. 2019). According to taxonomic keys, the cyanobacteria species were determined based on cell structure, colony morphology and mucilage characteristics. Since the toxic and non-toxic cyanobacterial species can co-exist in an environment and are indistinguishable by microscopy technique (Romanis et al. 2021), therefore, MCs producers were characterized based on the molecular approach (Casero et al. 2019) and MCs detection (Kumar et al. 2020b).

Molecular characterization of uncultured cyanobacteria in water samples of river Ganga

The *mcy* genes (*mcyA* to *mcyJ*) that regulate MCs synthesis in potentially toxic cyanobacteria have been identified and sequenced (Dittmann and Borner 2005). MCs standard structure is cyclo (D-Ala-L-X-D-MeAsp-L-Y-Adda-D-Glu-Mdha), where X and Y are variable L-amino acids. Figure 2 shows

Table 3 Water Quality Index at 10 sampling sites of the river Ganga at Prayagraj and Varanasi using NSFQWI method

Duration	Prayagraj					Varanasi					Over all
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	
June 2017	49.46	46.89	45.61	46.74	47.01	43.87	45.25	48	45.8	44.49	46.31
March 2018	51.52	49.99	50.68	49.66	51.31	50.79	49.21	52.22	50.52	50.67	50.66

Sampling sites - PRAYAGRAJ: (S1) Allahabad Sangam,(S2) Daraganj Ghat area, (S3) Mehdhori Gaon Kachar,(S4) Rasoolabad Ghat, (S5) Shastri Bridge area; VARANASI: (S6) Dashaswamegh Ghat, (S7) Ramana Village (S8) Ravidash mandir Ghat,(S9) Samne Ghat, (S10) Sheetla Ghat. Excellent: 90–100, Good: 70–90, Medium: 50–70, Bad: 25–50, and very bad: 0–25

the structure of MC-LR, where X is L-leucine and Y is L-arginine. In this study, PCR was conducted with general and genus-specific primers for three genes (*mcyA*, *mcyB* and *mcyE*) designed (Fig. 2) to amplify the generic *mcyE* (812 bp), *Microcystis mcyE* (247 bp), *Anabaena mcyE* (244 bp), *Planktothrix mcyE* (249 bp), *mcyA* (291 bp) and *mcyB* (973 bp) genes from phytoplanktonic uncultured water samples of river Ganga from each of the 10 sampling sites of Varanasi and Prayagraj regions. The PCR amplification with primers for the *Anabaena* and *Planktothrix mcyE* failed to produce any amplicons (data not shown). The result indicates that *Anabaena* and *Planktothrix* cells if present in the collected phytoplanktonic water samples were non-toxic due to absence of *mcy* genes. However, the desired length of PCR products was amplified using the oligonucleotide primers sets for generic *mcyE*, *Microcystis mcyE*, *mcyA* and *mcyB* genes from all the genomic DNA of the uncultured cyanobacteria extracted individually from water samples of 10 different sites as shown in Fig. 4. These findings of PCR amplification were same for both the years of study. Previous studies have also shown the identification and detection of toxic cyanobacteria in environmental samples based on *mcy* genes using a PCR technique (Baker et al. 2002, Ribeiro et al. 2020) with universal primers targeting conserved sequences (Hisbergues et al. 2003) and species-specific primers designed based on differences within the *mcy* gene clusters (Rantala et al. 2006), respectively. The *mcy* genes preferably targeted are *mcyA*, B, C, D, and E; however, in several studies, a combination of the *mcy* genes was used (Ouellette et al. 2006). The *mcyA* gene, because of its conserved sequences, is an appropriate target for PCR based identification of toxic cyanobacteria (Hisbergues et al. 2003; Rantala et al. 2004). The *mcyB* gene regulates the activation of amino acids as aminoacyl adenylate and it was followed by peptide bond formation by the condensation domain in growing MC molecule (Tillett et al. 2000). The glutamate-activating adenylation domain is encoded by *mcyE* gene and all known MC variants possess D-glutamate and the carboxyl group of the glutamate side-chain is crucial for the toxicity. Therefore, the *mcyE* gene used for detection of MCs-producing cyanobacteria (Goldberg et al. 1995). The amplicons of generic *mcyE*, *mcyA* and *mcyB* genes from uncultured cells of Ganga water samples were sequenced and submitted in the NCBI database. Gene bank accession ID numbers are MZ222414, MZ222415, and MZ222414 for *mcyE*, *mcyA*, and *mcyB* genes, respectively. The partial gene sequence similarities for *mcyA* and *mcyE* genes from uncultured cells of water samples with the genus *Microcystis* were more than 95 %. The neighbor-joining tree developed using *mcyA* and *mcyE* gene sequences showed the grouping with cyanobacterial genus *Microcystis* (Fig. 4). These results are consistent with the microscopic analysis and advocate the presence of genus *Microcystis* in river Ganga. However, based on sequenced *mcyB* amplicons from uncultured cells, the

phylogenetic assessment suggested close proximity with filamentous cyanobacterial genus *Oscillatoria* and *Planktothrix*. Thus, PCR amplification and phylogenetic analysis propose the presence of various genera of cyanobacteria possessing putative *mcy* genes in river Ganga.

Quantification of MC-LR equivalence in Ganga water by PPIA and HPLC method

Increasing pollution in river Ganga due to anthropogenic activities triggers MCs producers and MCs concentration in the water (Dixit et al. 2017). A study indicates that MCs that produced intracellularly have released into the extracellular environments during cell lysis due to stress or age (Wei et al. 2020). MC-LR is one of the most common and toxic MCs (Zhang et al. 2021). In this work, the MC-LR equivalence concentration in phytoplanktonic water samples collected from 10 different sampling points of Varanasi and Prayagraj regions was detected by PPIA and HPLC methods. The colorimetric PPIA technique determined the MC-LR equivalence concentration in river Ganga at study sites attaining low detection (LOD) even below 1 µg/L (Supplementary Fig. 2). PPIA method ensures the presence of MC-LR equivalence in all the collected Ganga water samples (Fig. 5). The HPLC method's calibration curve for MC-LR was built considering the areas of the chromatographic peaks calculated at its six different concentration levels, ranging from 0.1 to 500 µg/L. The good linearity was obtained for the regression equation of the calibration curve of HPLC analysis and it was $Y = 196.12X$ ($R^2 = 0.98$), where Y is the area of MC-LR peak and X is the concentration of the MC-LR in µg/L. The limit of detection (S/N=3) and limit of quantification (S/N=5) was 0.5 µg/L and 1.1 µg/L, respectively. The range of MC-LR equivalence concentration was observed to be 23–172 ng/L by PPIA, whereas, MC-LR equivalence concentration range was obtained to be 13–97 ng/L using HPLC at various sites of Varanasi and Prayagraj. The results of the actual MC-LR equivalence concentration obtained in water samples by the two mentioned methods are summarized in Fig. 5. The MC-LR equivalence concentrations observed by PPIA slightly differ from the HPLC values; however, still being of the same order of magnitude (Fig. 5). This difference may be possible due to the presence of compounds in river Ganga water that interferes on the PPIA, despite the high dilutions used (Garibo et al. 2014; Chen et al. 2005). These analyses further confirm the occurrence of putative MCs producing cyanobacterial strains in the Ganga water at Prayagraj and Varanasi regions. The concentration of MC-LR equivalence (≥ 60 ng/L) was high at Rasoolabad Ghat (S4), Daraganj Ghat (S2) and Mehdori Village (S3) in Prayagraj, where human activities are prevalent. In Varanasi, MC-LR equivalence was higher at Dashashwamegh Ghat (S6) and Ravidash

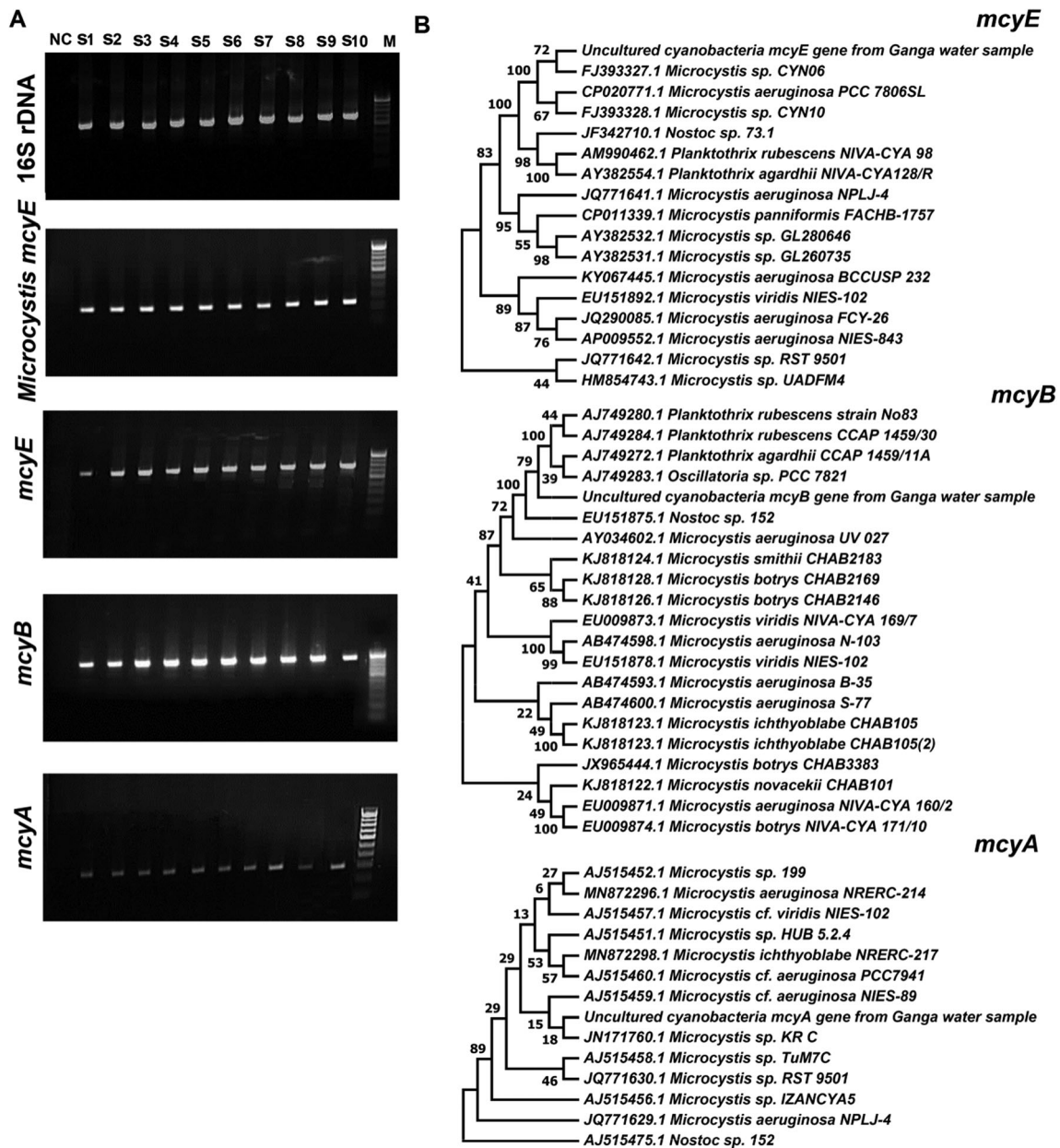


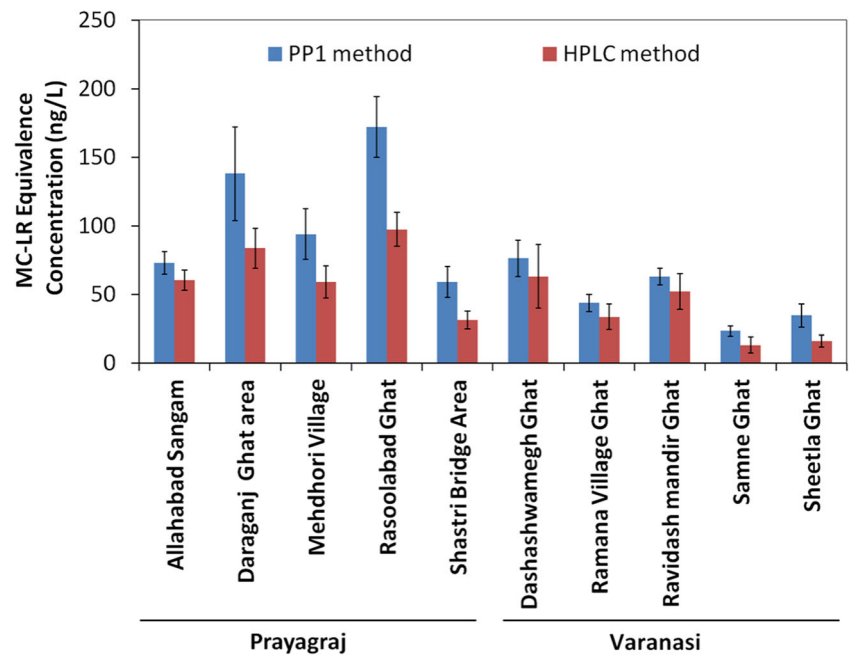
Fig. 4 A) PCR amplification of 16S rDNA and microcystin synthetase (*mcy*) genes of uncultured cyanobacteria in phytoplanktonic water samples of river Ganga collected from 10 different sampling sites of Prayagraj and Varanasi. (NC-negative control, S1-Allahabad Sangam, S2-Daraganj Ghat area, S3-Mehdhor Gaon Kachar, S4-Rasoolabad Ghat, S5-Shastri Bridge area, S6 –Dashaswamegh Ghat, S7-Ramana Village, S8-Ravidash mandir Ghat, S9-Samne Ghat, S10-Sheetla Ghat,

Mandir Ghat (S8). However, between two cities, MC-LR equivalence concentrations were generally higher in Prayagraj. The differences observed may reflect the influence of environmental factors on MCs production. The study demonstrated that MCs production is sensitive to several physico-chemical parameters, including light intensity, temperature, rainfall, pH, iron concentration, nitrogen, and phosphorus concentrations (Kaebnick and Neilan 2001, Wagner et al.

M – Marker). B) The evolutionary history was inferred based on *mcyA*, *mcyE* and *mcyB* gene sequences using the NJ method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Kimura 2-parameter method. Evolutionary analyses were conducted in MEGA6 software (Tamura et al. 2013)

2019). This is the first study that reported the quantitative MC-LR equivalence estimation in the water of river Ganga. However, MC-LR equivalence concentration is far less in river Ganga's water than the harmful exposure concentration (1 µg/L) recommended by WHO (WHO, 2011). PPIA is more convenient to perform and could be used as a primary method to detect MCs presence and its concentration in the water reservoirs (Massey et al. 2020).

Fig. 5 Concentration of MC-LR equivalence detected by PP1 inhibition assay and HPLC method in the river Ganga water samples collected during March 2018 from 10 different sampling sites of Prayagraj and Varanasi regions



Isolation and characterization of MC producing cyanobacteria from river Ganga

Three different cyanobacterial strains were isolated and purified by plating and liquid culture methods in BG-11 media. Visualization using phase contrast microscope showed that one of the isolates is the macroscopic, circular, colonial and gas vacuolated form (Ganga isolate 1), the second one is of non-colonial, filamentous structure (Ganga isolate 2) and the third one is the polymorphic colonial form (Ganga isolate 3)

(Supplementary Fig. 3). Of the three isolates, only Ganga isolate 1 amplifies the PCR products for *mcy* genes (Fig. 6). Initially, based on morphological identification, the Ganga isolate 1 was identified as *Microcystis*. Furthermore, the partial 16S rDNA fragment from Ganga isolate 1 was amplified using cyanobacterial specific universal primer. The amplicon was sequenced and submitted in the NCBI database. Gene bank accession ID number is MZ027619. The BLAST hit of partial 16S rDNA gene sequence of Ganga isolate 1 indicates its maximum phylogenetic identity with *Microcystis*

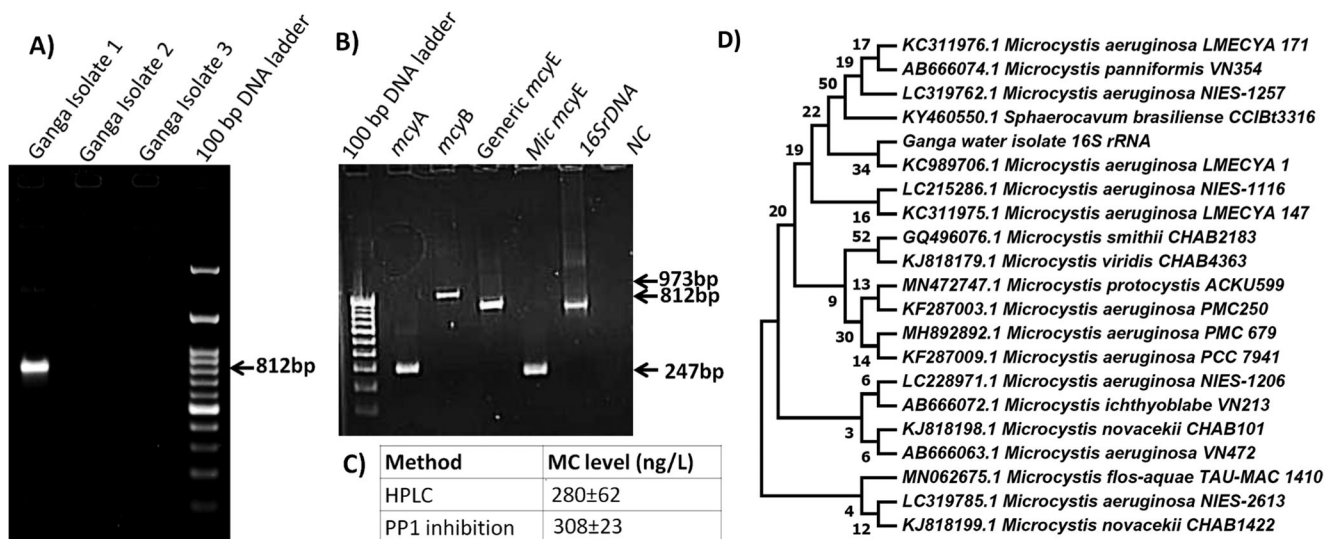


Fig. 6 PCR amplification of **A)** generic *mcyE* gene from three different Ganga isolate, **B)** 16S rDNA and microcystin synthase (*mcy*) genes from Ganga isolate 1, **C)** microcystin-LR concentration by Ganga isolate 1, and **D)** The evolutionary history was inferred based on partial 16S rDNA sequence of Ganga isolate 1 using the NJ method. The bootstrap

consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Kimura 2-parameter method. Evolutionary analyses were conducted in MEGA6 software (Tamura et al. 2013)

aeruginosa LMECYA 1 (Fig. 6). Results confirmed that the isolate was *Microcystis*. Thus the result of morphological identification is supported by phylogenetic analysis based on partial 16S rDNA sequence as shown in Fig. 6. Only one of the three isolated cultures, i.e., Ganga isolate 1 (*Microcystis*) from river Ganga was a MCs producer based on PPIA and HPLC method (Fig. 6). The majority of the MCs positive water bodies were eutrophic and turbid. The researcher also revealed that physico-chemical factors directly or indirectly affect cell growth and MCs generation in water bodies (Bouaïcha et al. 2019). In this study, not much correlation has been drawn between the physico-chemical parameters and MC-LR equivalence concentration at the sampling sites; therefore, it is impossible to reliably determine the cause for putative MCs producers in river Ganga. Though, a weak positive influence of phosphate, ammonium, and temperature has been observed. In the future, extensive research with more sampling and a more extended monitoring period is needed to elucidate the relative importance and correlation.

Conclusion

The polyphasic approach was applied to ascertain the water quality at recreational sites of the river Ganga flowing in the stretch from Prayagraj to Varanasi by assessing physico-chemical, WQI analysis combined with toxicological characters such as MCs producers based on the generic and species-specific primers (*mcyE*, A and B) and MC-LR equivalence level detection. Physico-chemical and WQI analysis indicates that the water quality at recreational sites is in an alarming situation. This study provides the first evidence about the prevalence of putative MCs producing cyanobacteria with MC-LR equivalence concentration below the permissible limit of WHO. The amount and variability of MC-LR equivalence concentrations may be related to variability in nutrient enrichment at different sites. Ganga isolate (named as Ganga isolate 1) was identified to be *Microcystis*, possessing *mcy* genes with MCs production potential. Higher alkalinity, turbidity and temperature may favor the growth of MCs producing cyanobacteria in river Ganga. Human populations and domestic animals living in this region may appear to be exposed to MCs through contaminated dietary components and/or drinking water. Hence, it is essential to perform the periodic assessment of MC producers and their toxins to reduce the chances of water-borne diseases, ensure good health for maintenance and restoration of the river Ganga water sanctity.

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Author contribution VK conceived and designed the experiments, performed the microscopic study and overall analysis of the data. VK and SK performed the sampling, physico-chemical analysis, phylogenetic tree analyses and interpreted the results. SK and IY performed the PPIA and HPLC experiments. VK, AC and SR performed the PCR experiments. VK and SP performed the cyanobacteria isolation and its maintenance. VK and SK wrote the paper.

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Data availability NA

Declarations This submitted work has not been published previously (except in the form of a project reports), and is not under consideration for publication elsewhere, it's publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright holder.

Ethics approval and consent to participate NA

Competing interests

The authors declare no competing interests.

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