

Effects of toxic *Microcystis* genotypes on natural colony formation and mechanism involved

Xiangdong Bi, Wei Dai, Shulin Zhang, Shaojie Dong and Dajuan Zhang

ABSTRACT

The cyanobacterium *Microcystis* occurs as colonies of different sizes with varying abundance of toxic genotypes versus non-toxic genotypes under natural conditions. To investigate the effects of toxic *Microcystis* genotypes on natural colony formation, samples collected from the mainstream of Haihe River from July to October 2015 were sieved into four colony classes with sizes of <8 µm, 8–20 µm, 20–90 µm and >90 µm. Each colony size class was analyzed for the proportion of toxic *Microcystis* genotypes, and microcystins (MCs) cellular production of toxic genotypes. The results showed the smallest size class of *Microcystis* colonies (<8 µm) showed the lowest proportion of toxic genotypes and the highest MC-RR and MC-YR cellular production. With the increasing colony sizes, the proportion of toxic *Microcystis* genotypes increased but the MC-RR and MC-YR cellular production decreased. A negative correlation between the MCs cellular production and the proportion of toxic genotypes was observed in all four colony size classes, suggesting that the less there were toxic *Microcystis* cells able to produce MCs, the more each toxic cell needed to produce that molecule. Toxic *Microcystis* played an important role in the colony formation in natural waters via producing MCs.

Key words | colony formation, Haihe River, microcystin, toxic *Microcystis*

Xiangdong Bi
Wei Dai (corresponding author)
Shulin Zhang
Shaojie Dong
Dajuan Zhang
Key Laboratory of Aquatic-Ecology and
Aquaculture of Tianjin, Department of Fisheries
Sciences,
Tianjin Agricultural University,
Tianjin 300384,
China
E-mail: daiweitj@126.com

Xiangdong Bi
MOE Key Laboratory of Pollution Processes and
Environmental Criteria, Tianjin Key Laboratory
of Environmental Remediation and Pollution
Control, College of Environmental Science and
Engineering,
Nankai University,
Tianjin 300071,
China

INTRODUCTION

Microcystis is a globally distributed bloom-forming genus of cyanobacteria (Haande *et al.* 2007; Shen & Song 2007). In natural waters, *Microcystis* cells are enveloped by an amorphous mucilage sheath and form colonies with different sizes and morphologies (Haider *et al.* 2003; Wang *et al.* 2015). Colony formation can protect *Microcystis* cells from zooplankton grazing, viral or bacterial attack, desiccation and other potential negative environmental factors, and increase floating velocity, all of which provide a competitive advantage over other phytoplankton genera (Kearns & Hunter 2000; Wu *et al.* 2007; Xiao *et al.* 2012; Yang & Kong 2012). However, the mechanisms underlying how and why *Microcystis* form colonies remain poorly understood.

When *Microcystis* colonies isolated from the natural waters are cultured in the laboratory, their colonial

phenotype is hard to maintain and always disaggregates into unicellular cells (Bolch & Blackburn 1996; Yang *et al.* 2006). This morphological variation is apparently related to the lack of some environmental factors that stimulate colony formation in laboratory cultures (Li & Dai 2016), and these environmental factors may be contributing to colony formation in the natural conditions. Various biotic and abiotic factors, such as zooplankton grazing pressure (Yang *et al.* 2008), heterotrophic bacteria co-cultivation (Shen *et al.* 2011), fluid motion (Li *et al.* 2013a), ultraviolet radiation (Quesada & Vincent 1997), high calcium levels (Wang *et al.* 2011), elevated heavy metal concentrations (Bi *et al.* 2013), low temperature (Trainor 1993) and illumination (Li *et al.* 2013b), have been identified to be stimulators of *Microcystis* colony formation in laboratory experiments.

Co-culture investigations have proved that environmental factors could affect the selection of toxic and non-toxic genotypes, and toxic cells always dominated in the growth-limiting conditions (Dziallas & Grossart 2011; Leblanc Renaud *et al.* 2011; Lei *et al.* 2015). Toxic *Microcystis*

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genotypes containing microcystin synthetase (*mcy*) gene cluster could produce and secrete microcystins (MCs) (Tillett et al. 2000). Gan et al. (2012) found that addition of MCs led to increased *Microcystis* colony size, while depletion of MCs caused colony size to decrease. It was suggested that MCs released by toxic genotypes acted as an internal factor that played a pivotal role in the development of *Microcystis* colonies. This knowledge, however, was obtained via laboratory experiment and needed to be proved in natural conditions. Toxic genotypes always coexist with non-toxic genotypes in natural *Microcystis* colonies (Davis et al. 2009; Li et al. 2012). Kurmayer et al. (2003) found that the proportion of toxic *Microcystis* genotypes varied within colonies in Lake Wannsee (Germany). The abundance and proportion of toxic *Microcystis* genotypes always varies in spatial and temporal scales in different freshwater systems (Li et al. 2014). More field data from a range of freshwater ecosystems are needed to assess the effects of toxic genotypes and MCs production on wild *Microcystis* colony formation.

Haihe River is the largest water system in North China. Its mainstream flows through Tianjin City and empties into Bohai Bay (Wang et al. 2014). The mainstream of Haihe River is an important urban emergency reserve water source, and also acts as a flood drainage channel in Tianjin City (Dai & Yu 2014). However, it has been seriously polluted by industrial, agricultural and domestic effluents (Zhao et al. 2014), and suffers annual cyanobacterial blooms dominated by *Microcystis* during summer (Zhou et al. 2013).

In this study, a field investigation, including the proportion of toxic *Microcystis* genotypes and MC cellular production of toxic genotypes in four different colony size

classes, was conducted in the mainstream of Haihe River. These findings are helpful in elucidating the relationship between the proportion of toxic *Microcystis* genotypes and colony formation, as well as MCs cellular production of toxic genotypes and colony formation during blooms.

MATERIAL AND METHODS

Sampling

A total of five sites were selected for water sampling in the mainstream of Haihe River in the Tianjin urban area. They were located in Jingang Bridge (JG, site N39°15'52.67", E117°19'82.22"), Guanghua Bridge (GH, site N39°10'53.62", E117°24'70.73"), Waihuan Bridge (WH, site N39°07'66.31", E117°32'33.25"), Xijian Floodgate (XJ, site N39°06'07.48", E117°38'78.73"), and Erdao Floodgate (ED, site N39°02'74.98", E117°46'84.52"), respectively (Figure 1). Sampling was performed monthly from July to October in 2015. At each sampling site, water samples were taken from the surface to the sediment using a cylindrical organic glass sampler with inner diameter of 10 cm. The sampler was designed and made by ourselves. Before the sampler was used for the next sampling site, it was rinsed with sterile BG11 medium. The volume of collected water varied with the sampling depth, which ranged from 1.5 to 3 m and changed according to the river depth. Water samples were collected for more than three times per sampling site, and then integrated before being transferred to the laboratory within no more than 2 h.

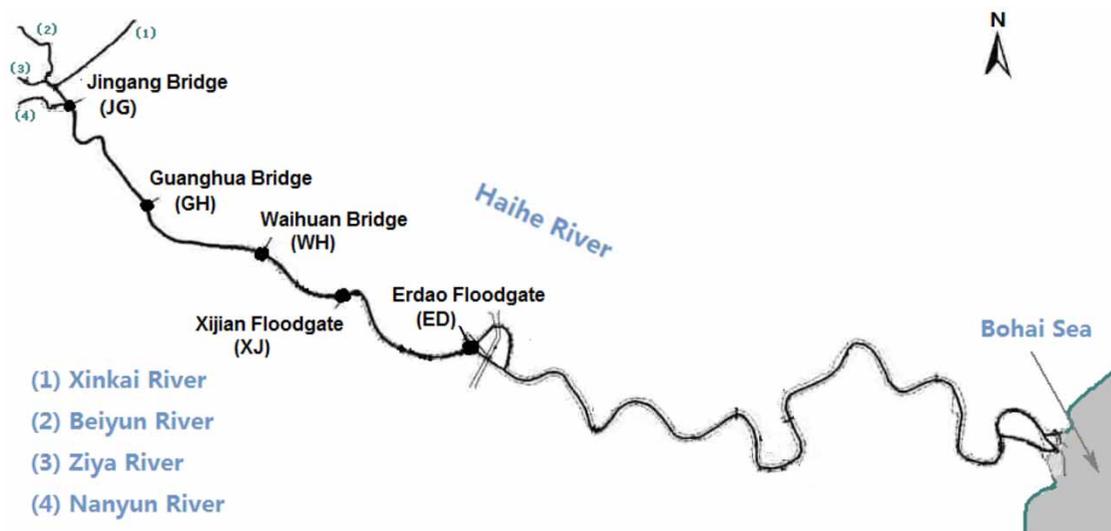


Figure 1 | Water sampling sites in the mainstream of Haihe River in Tianjin urban area.

Morphospecies identification, cell number determination and colony separation

1 L of integrated water sampled per sampling site was fixed with Lugol's solution. Phytoplankton species were identified according to the methods of Hu & Wei (2006) and counted using a blood counting chamber under microscope ($\times 40$). *Microcystis* colonies were separated before being counted according to the method of Bi et al. (Bi et al. 2015). To separate super-large colonies ($>90\ \mu\text{m}$), large colonies ($20\text{--}90\ \mu\text{m}$), middle colonies ($8\text{--}20\ \mu\text{m}$) and small colonies ($0.45\text{--}8\ \mu\text{m}$), water samples were poured gently through sieves (Lingyun Rubber and Plastic Co., Ltd) with four different mesh sizes ($90, 20, 8$ and $0.45\ \mu\text{m}$) within minutes (Yang et al. 2009; Bi et al. 2015).

DNA extraction

Water samples and separated algal cell suspensions of each colony size class were filtered with GF/C filters, respectively. DNA extraction was performed according to the protocol of Rinta-Kanto (Rinta-Kanto et al. 2005). Briefly, GF/C filters cut into pieces were subjected to lysis buffer ($40\ \text{mM}$ EDTA– $400\ \text{mM}$ NaCl– $50\ \text{mM}$ Tris–HCl [pH 9.0]). Cell lysis was achieved by the addition of lysozyme ($2\ \text{mg mL}^{-1}$, $37\ ^\circ\text{C}$, 30 min) and subsequently proteinase K ($100\ \mu\text{g mL}^{-1}$ in 2% sodium dodecyl sulfate, $50\ ^\circ\text{C}$, 2–3 h). After extraction with phenol–chloroform–isoamyl alcohol (25:24:1 [V/V/V]) once and chloroform–isoamyl alcohol (24:1 [V/V]) twice, DNA was precipitated using 100% (V/V) ethanol and $10\ \text{mol L}^{-1}$ ammonium acetate solution ($-20\ ^\circ\text{C}$, overnight). The purified DNA was resuspended in $100\ \mu\text{L}$ of Millipore water after washing with 70% (V/V) ethanol.

Quantitative real-time PCR

The real-time polymerase chain reaction (PCR) assay was used to quantify two genetic elements, the *PC-IGS* and *mcyA* regions. The *Microcystis PC-IGS* region, which is specific to the *Microcystis* genus, was used to quantify the abundance of the total *Microcystis* population (Kurmayer & Kutzenberger 2003). The *mcyA* gene found within the microcystin synthetase gene operon, which only appears in toxic genotypes of *Microcystis*, was used to quantify the toxic *Microcystis* population (Yoshida et al. 2007). *PC-IGS* was amplified using 188F ($5\text{'-GCT ACT TCG ACC GCG CC-3'}$) and 254R ($5\text{'-TCC TAC GGT TTA ATT GAG ACT AGC C-3'}$) primers (Chen et al. 2003), and the *mcyA* gene was amplified using M1r-F ($5\text{'-AGC GGT AGT CAT TGC ATC GG-3'}$) and M1r-R ($5\text{'-GCCCTT TTT CTG AAG TCG CC-3'}$) primers (Yoshida et al. 2007).

Standards used to determine the *PC-IGS* and *mcyA* gene copy numbers were prepared using the genomic DNA of *M. aeruginosa* strain PCC7806 provided by the FACHB Collection (Freshwater Algal Culture Collection of Institute of Hydrobiology, China). Algal cells from a known volume of the *M. aeruginosa* 7806 culture were filtered through $0.22\ \mu\text{m}$ membranes, and the DNA was extracted as described above. The DNA concentration (ABS260 nm) and purity (ABS260 nm/ABS280 nm) were determined spectrophotometrically. The approximate genome size of *M. aeruginosa* 7806 is 5.2 Mb (Frangeul et al. 2008), and the average molecular weight of 1 bp was $660\ \text{g mol}^{-1}$ (Vaitomaa et al. 2003). Copy numbers of both genes on the basis of DNA were evaluated according to the method of Vaitomaa et al. (2003). A 10-fold dilution series of the DNAs was prepared and amplified with the *PC-IGS* and *mcyA* real-time PCR assays. Linear regression equations for obtained cycle threshold values (Ct values) were then calculated as a function of known *mcyA* and *PC-IGS* copy numbers according to Vaitomaa et al. (2003).

The real-time PCR was performed in a volume of $25\ \mu\text{L}$ reaction mixture containing $12.5\ \mu\text{L}$ $2\times$ SYBR Premix EX Taq™ (TaKaRa, Japan), $1.0\ \text{pmol}$ of each primer, $2\ \mu\text{L}$ DNA from a standard strain or $2\ \mu\text{L}$ DNA from water sample, and filled up to $25\ \mu\text{L}$ with sterile ultra-pure water. The PCR was carried out on the Mastercycler realplex 4 system (Eppendorf, Germany) with Mastercycler realplex software. Amplification of *PC-IGS* was performed as follows: initial denaturation at $95\ ^\circ\text{C}$ for 2 min, followed by 40 cycles at $95\ ^\circ\text{C}$ for 20 s, annealing at $56\ ^\circ\text{C}$ for 30 s, and elongation at $72\ ^\circ\text{C}$ for 30 s. For *mcyA*, the amplification included initial denaturation at $95\ ^\circ\text{C}$ for 2 min, followed by 40 cycles: $95\ ^\circ\text{C}$ for 20 s, $58\ ^\circ\text{C}$ for 30 s, and $72\ ^\circ\text{C}$ for 20 s. The melting temperature for the real-time PCR products was determined using the manufacturer's software. All of the samples were amplified in triplicate.

MCs analysis

For intracellular MCs analysis, the algal cell suspensions of each colony size class were centrifuged first ($10,000\ \text{rpm}$, 10 min) at $4\ ^\circ\text{C}$, and then subjected to repeated freezing ($-20\ ^\circ\text{C}$) and thawing ($4\ ^\circ\text{C}$). After centrifugation, the supernatant I was collected and the cell pellet was stirred with 5% (V/V) acetic acid for 20 min, and then centrifuged to collect supernatant II. The left cell pellet was stirred with 80% (V/V) aqueous methanol for 20 min, and then centrifuged to collect supernatant III. The methanol left in supernatant III was removed via rotary evaporation. The supernatant I, II and III were mixed and filtered through a $0.22\ \mu\text{m}$ membrane to prepare filtration for the subsequent solid phase extraction (SPE).

MCs were extracted using an SPE C₁₈ cartridge, which yielded 78.53% and 81.49% recovery for MC-RR and MC-YR, respectively. The SPE C₁₈ cartridge activated with methanol was cleaned using distilled water, and then loaded with filtration prepared. The flow rate was 8–12 mL/min. After washed with distilled water and 20% (V/V) aqueous methanol in sequence, MCs was eluted using 60% (V/V) aqueous methanol (with 0.05% [V/V] trifluoroacetic acid). The elute collected was rotary evaporated to dryness, redissolved in 60% (V/V) aqueous methanol, and then filtrated through a 0.22 µm membrane prior to high-performance liquid chromatography (HPLC) analysis.

Extracted MCs were analyzed using HPLC System (SPD-M20A, Shimadzu, Japan) equipped with a Shim-Pack VP-ODS column (250 mm × 4.6 mm), using 60% aqueous methanol (with 0.05% (V/V) trifluoroacetic acid) at a flow rate of 1 mL/min. MCs (MC-RR and MC-YR) were identified using their characteristic absorption spectra. Certified standards of MC-RR and MC-YR (Sigma, USA) were used to create standard curves.

Statistical analysis

On the same sampling date and sampling site, relationships between MCs (MC-RR, MC-YR and sum of MC-RR and MC-YR) cellular production of toxic *Microcystis* genotypes and the proportion of toxic *Microcystis* genotypes in four colony size classes were analyzed using Pearson's correlation. All statistical tests were conducted by statistical software (SPSS Version 17.0) and $P < 0.05$ was considered significant.

RESULTS

The proportion of *Microcystis* cells in four colony size classes to the total *Microcystis* cells

All four colony size classes were found throughout the study period. Phytoplankton identification of water samples revealed that two *Microcystis* morphospecies (*M. aeruginosa* and *M. novacekii*) (Figure 2) dominated the phytoplankton biovolume from July to October in the mainstream of Haihe River (Table 1).

From the standard curves for *PC-IGS* and the *mcyA* gene, two highly significant linear relationships were observed between the threshold value and the log value of the template DNA copy numbers when the genomic DNA from *M. aeruginosa* PCC7806 was used as template

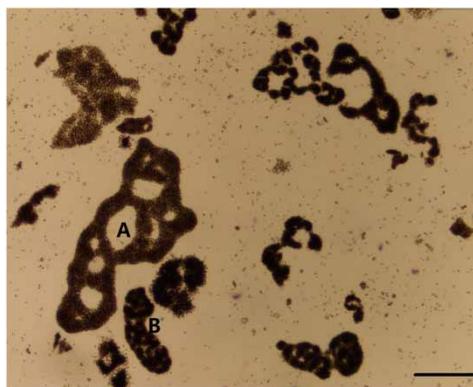


Figure 2 | *Microcystis* morphospecies occurred in the mainstream of Haihe River. (A) *M. aeruginosa*, (B) *M. novacekii*, scale bars indicates 50 µm.

(Figure 3). As shown in Figure 4, there was significant difference in the proportion of cells carrying the *PC-IGS* gene in four colony size classes to the total cells carrying the *PC-IGS* gene. The larger size classes of *Microcystis* colonies (20–90 µm or >90 µm) contributed the most to the total *Microcystis* cells. The colony with a size of 8–20 µm contributed the least to the total *Microcystis* cells on each sampling date, with the exception of October 4, on which day the colony with a size of <8 µm contributed the lowest proportion.

Proportion of toxic *Microcystis* genotype cells to total *Microcystis* cells in four colony size classes

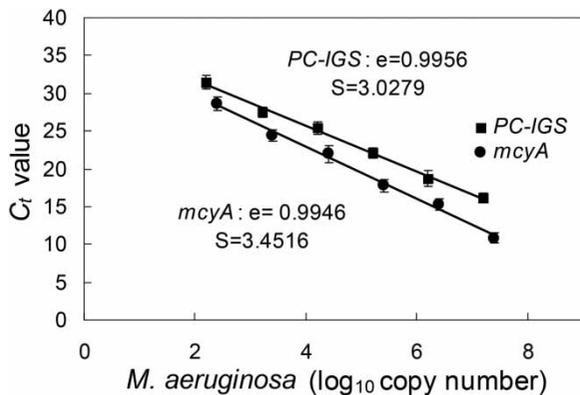
The proportion of toxic *Microcystis* genotypes calculated using the ratio of the *mcyA* gene to the *PC-IGS* gene varied with sampling dates, ranging from 3.25 to 23.98, from 1.27 to 8.00, from 3.21 to 13.39, and from 0.82 to 11.23% in July, August, September, and October, respectively (Table 1). The proportion of toxic *Microcystis* genotypes in four colony size classes is shown in Figure 5. The larger colonies had a higher proportion of toxic genotypes. The colony with a size of 20–90 µm showed a much higher proportion of toxic *Microcystis* in July, while the highest toxic proportion appeared in colonies with a size of 20–90 µm or >90 µm from August to October. Over the entire study period, the smallest colony size class (<8 µm) showed the lowest toxic proportion at all five sampling sites.

MCs cellular production of toxic *Microcystis* genotypes in four colony size classes

MCs cellular production of toxic *Microcystis* genotypes were expressed as microgramme (µg) equivalents of MC

Table 1 | The proportion of *Microcystis* in total phytoplankton abundance (A) and the proportion of toxic *Microcystis* genotypes cells to total *Microcystis* cells (B)

Sampling date	Sampling site									
	ED		XJ		WH		GH		JG	
	A	B	A	B	A	B	A	B	A	B
26 Jul 15	82.19	3.25	96.50	3.98	91.86	9.01	56.93	23.90	45.78	23.98
20 Aug 15	87.96	1.27	74.82	8.00	100	8.77	64.87	2.90	45.56	2.85
12 Sep 15	49.68	4.21	37.48	3.21	93.35	8.32	57.19	13.39	36.83	11.71
4 Oct 15	50.32	0.82	93.25	2.08	95.95	2.60	64.68	6.77	32.85	11.23

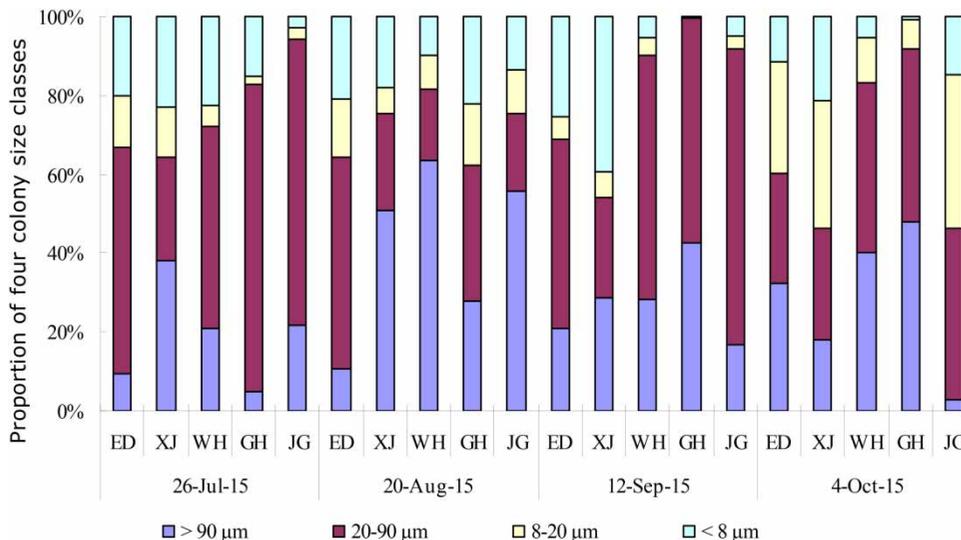
**Figure 3** | Standard curves obtained by the *PC-IGS* gene and *mcyA* real-time PCR assays with the *M. aeruginosa* strain PCC7806 as a function of gene copy numbers. Each data point shows the threshold cycle (C_t) of standard DNA samples performed in triplicate. Amplification efficiency was calculated as follows: $e = 10^{-1/S} - 1$, where S is the slope. Error bars represent the standard deviations.

per 10^8 copies of the *mcyA* gene in different colony size classes. As shown in Table 2, MC-RR cellular production of toxic *Microcystis* genotypes in the mainstream of

Haihe River varied with sampling dates. On each sampling date, MC-RR cellular production of toxic *Microcystis* genotypes decreased with increasing colony sizes at each sampling site, and MC-YR cellular production of toxic *Microcystis* genotypes presented the same change tendency (Table 3). Both the highest MC-RR and MC-YR cellular productions were found in colonies with a size of $<8 \mu\text{m}$, followed by colonies with sizes of $8\text{--}20 \mu\text{m}$, $20\text{--}90 \mu\text{m}$ and $>90 \mu\text{m}$.

Correlation analysis

On each sampling date and at each sampling site, MCs (MC-RR, MC-YR and the sum of MC-RR and MC-YR) cellular production of toxic *Microcystis* genotypes in each colony size class was negatively correlated with the proportion of toxic *Microcystis* genotypes in each colony size class (Table 4). All coefficient values were below -0.57 except the one that was found at GH on August 15.

**Figure 4** | The proportion of *Microcystis* cells in four colony size classes to the total *Microcystis* cells (%).

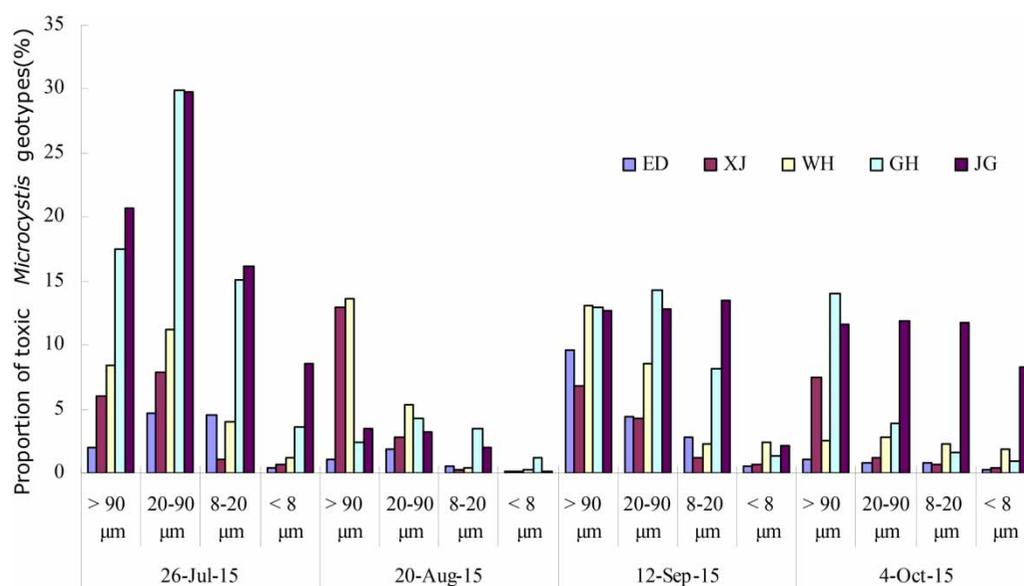


Figure 5 | Proportion of toxic *Microcystis* genotype cells to total *Microcystis* cells in four colony size classes (%).

Table 2 | MC-RR cellular production of toxic *Microcystis* genotypes in four colony size classes ($\mu\text{g } 10^8 \text{ copies}^{-1}$)

Sampling date	Colony size	Sampling site				
		ED	XJ	WH	GH	JG
26 Jul 15	>90 μm	1.44	1.09	1.02	4.95	22.17
	20–90 μm	2.85	2.89	1.50	53.46	63.14
	8–20 μm	10.49	19.76	3.60	132.89	113.67
	<8 μm	31.41	32.32	23.75	141.06	146.05
20 Aug 15	>90 μm	19.05	4.77	1.55	0.04	38.29
	20–90 μm	34.55	25.47	5.25	14.47	56.15
	8–20 μm	69.04	98.39	59.06	20.04	114.02
	<8 μm	163.66	186.37	138.23	28.15	136.86
12 Sep 15	>90 μm	0.04	6.20	3.10	28.84	9.96
	20–90 μm	9.44	14.97	4.85	21.04	11.71
	8–20 μm	56.60	48.47	31.42	125.27	86.25
	<8 μm	90.13	114.14	66.15	171.13	93.01
4 Oct 15	>90 μm	0.04	0.04	25.58	7.43	38.54
	20–90 μm	10.43	14.66	45.24	20.67	86.27
	8–20 μm	32.87	22.75	62.25	39.24	116.21
	<8 μm	71.52	44.23	93.62	71.69	167.89

DISCUSSION

During 2015, the mainstream of Haihe River hosted cyanobacterial blooms dominated by *Microcystis* from July to October. Consistent with previous reports (Davis et al. 2009; Li et al. 2012), we found that toxic *Microcystis* genotypes coexisted with non-toxic genotypes during *Microcystis* blooms. Toxic *Microcystis* cells comprised

between 0.82% and 23.98% of the total *Microcystis* population at five sampling sites, a range smaller than 4.4–64.3% in Lake Taihu (China) (Li et al. 2012) and 0.5–35% in Lake Mikata (Japan) (Yoshida et al. 2007). The lower proportion of toxic *Microcystis* (0.01–0.6%) was found in Lake Champlain, Lake Agawam, and Mill pond (USA) (Davis et al. 2009). In these freshwater ecosystems, dominance of non-toxic *Microcystis* was a more general phenomenon.

Table 3 | MC-YR cellular production of toxic *Microcystis* genotypes in four colony size classes ($\mu\text{g } 10^8 \text{ copies}^{-1}$)

Sampling date	Colony size	Sampling site				
		ED	XJ	WH	GH	JG
26 Jul 15	>90 μm	5.33	3.64	8.46	1.27	33.05
	20–90 μm	8.40	9.32	9.40	6.20	59.04
	8–20 μm	29.59	51.58	14.56	32.34	152.19
	<8 μm	60.60	91.53	78.73	97.39	167.75
20 Aug 15	>90 μm	1.27	2.95	2.89	1.89	1.27
	20–90 μm	1.27	20.54	12.32	6.63	12.41
	8–20 μm	1.89	131.46	80.40	7.20	21.62
	<8 μm	4.97	178.92	115.99	8.52	39.71
12 Sep 15	>90 μm	10.84	7.69	5.12	1.89	2.03
	20–90 μm	11.53	7.78	4.69	6.87	4.77
	8–20 μm	23.01	19.08	18.14	8.52	12.32
	<8 μm	66.22	69.96	38.57	11.26	29.59
4 Oct 15	>90 μm	9.50	1.27	1.89	2.89	1.27
	20–90 μm	27.94	24.07	5.06	2.47	6.87
	8–20 μm	47.05	35.68	21.67	16.37	11.53
	<8 μm	96.53	88.96	62.21	29.64	14.56

Table 4 | Pearson's correlation coefficient (r) of MCs (MC-RR, MC-YR and sum of MC-RR and MC-YR) cellular production of toxic *Microcystis* genotypes to the proportion of toxic *Microcystis* genotype cells to total *Microcystis* cells in each colony size class

Sampling date	Sampling site									
	ED		XJ		WH		GH		JG	
	r	P	r	P	r	P	r	P	r	P
MC-RR										
26 Jul 15	-0.67	0.33	-0.92	0.08	-0.80	0.20	-0.61	0.39	-0.73	0.27
20 Aug 15	-0.76	0.23	-0.73	0.27	-0.74	0.26	-0.28	0.72	-0.94	0.06
12 Sep 15	-0.88	0.12	-0.85	0.15	-0.82	0.17	-0.97*	0.03	-0.57	0.43
4 Oct 15	-0.97*	0.03	-0.80	0.20	-0.83	0.17	-0.78	0.22	-0.78	0.22
MC-YR										
26 Jul 15	-0.59	0.41	-0.90	0.10	-0.79	0.21	-0.85	0.15	-0.79	0.21
20 Aug 15	-0.79	0.21	-0.77	0.23	-0.85	0.15	-0.08	0.92	-0.98*	0.02
12 Sep 15	-0.76	0.24	-0.73	0.28	-0.79	0.21	-0.79	0.21	-0.91	0.09
4 Oct 15	-0.99*	0.01	-0.72	0.28	-0.91	0.09	-0.67	0.33	-0.66	0.34
MC-RR + MC-YR										
26 Jul 15	-0.62	0.38	-0.90	0.10	-0.79	0.21	-0.74	0.26	-0.77	0.23
20 Aug 15	-0.76	0.16	-0.75	0.25	-0.80	0.20	-0.25	0.76	-0.96*	0.04
12 Sep 15	-0.85	0.15	-0.81	0.19	-0.81	0.19	-0.97*	0.03	-0.66	0.34
4 Oct 15	-0.98*	0.02	-0.75	0.25	-0.88	0.12	-0.75	0.25	-0.77	0.23

*Correlation is significant at the 0.05 level.

It has been found that the proportion of toxic *Microcystis* varies greatly in different colony size classes. In Lake Taihu, the larger size colonies had a higher proportion of

potential toxic genotype among four *Microcystis* colonies with size of <50 μm , 50–100 μm , 100–270 μm and >270 μm . Kurmayer & Kutzenberger (2003) estimated the

proportion of toxic genotypes within *Microcystis* colonies to be 1.7–71% in Lake Wannsee (Germany). The larger colony size classes (>100 µm) showed the highest proportion of toxic genotypes, whereas in the smaller size classes of *Microcystis* colonies (<100 µm), toxic genotypes were less abundant (Kurmayer et al. 2003). This is in consistent with our results in spite of the fact that colonies were separated in different sizes in the mainstream of Haihe River. As compared to toxic genotypes, non-toxic *Microcystis* genotypes are better competitors for light (Kardinaal et al. 2007) and have a better fitness in low-light conditions (Sabart et al. 2013). Meanwhile, larger colonies are known to perform more pronounced vertical migration via buoyancy regulation than smaller colonies and are able to use light more efficiently than smaller colonies (Wallace et al. 2000). It could be potentially suggested that colony size and the proportion of toxic genotypes might be involved in competition for environmental resources, e.g. light. This hypothesis requires further testing.

In Lake Taihu, the MC cell quota calculated for all *Microcystis* cells tended to be higher during blooms in colonies with a larger size (Wang et al. 2013). Considering that non-toxic *Microcystis* cells have no MC-producing capacity, the MC cell quota calculated using the above method could not reflect the actual MC-producing ability of toxic cells. In the mainstream of Haihe River, MC cellular production was calculated only for the cells carrying the *mcyA* gene, and it tended to be higher in colonies with a smaller size. The mainstream of Haihe River, as an important urban emergency reserve water source for Tianjin City, is a semi-enclosed water body enclosed with floodgates (Dai & Yu 2014). When the mainstream of Haihe River suffered cyanobacterial blooms, regular water diversion from Luanhe River or Yuqiao Reservoir was adopted to replace cyanobacteria-polluted water (Tian 2015). Water replacement measure took away most surface water and large colonies, because large colonies have faster vertical migration (Nakamura et al. 1993) and mainly concentrate at the water surface to form blooms (Wu & Kong 2009). *Microcystis* cells in small-sized colonies were more susceptible to stress and grazing (Yang & Kong 2012), and less competitive for light and nutrients as compared to cells in larger-sized colonies (Wallace et al. 2000). To protect cells against competitors and/or predators, a small-sized colony needed to aggregate to a larger-sized colony. It was known that the extracellular polysaccharides (EPS) in the mucilage or sheath of *Microcystis* could contribute to colony formation. As demonstrated by the results of Gan et al. (2012), MCs could stimulate colony formation in *Microcystis* via upregulating

part of the polysaccharide biosynthesis-related genes. In the present study, we found that the smallest size class of *Microcystis* colony (<8 µm) showed the highest MCs cellular production of toxic *Microcystis*, suggesting that toxic *Microcystis* in a small-sized colony is needed to produce more MCs to stimulate the formation of a larger-sized *Microcystis* colony via increasing EPS production.

In all four colony size classes, there was a negative correlation between MCs (MC-RR, MC-YR, sum of MC-RR and MC-YR) cellular production of toxic *Microcystis* genotypes and the proportion of toxic *Microcystis* genotypes. With the increasing colony sizes, MCs cellular production decreased. The less there were toxic *Microcystis* cells able to produce MCs, the more each toxic cell needed to produce that molecule. It has been suggested that toxic *Microcystis* plays an important role in the colony formation via producing the intraspecific signaling molecules of MCs in the natural environment.

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