Available online at www.sciencedirect.com
ScienceDirect
Journal of Environmental Sciences 2012, 24(2) 284–290
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

Quantification of microcystin-producing and non-microcystin producing *Microcystis* populations during the 2009 and 2010 blooms in Lake Taihu using quantitative real-time PCR

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Received 18 March 2011; revised 03 May 2011; accepted 11 May 2011

Abstract

Lake Taihu, a large, shallow hypertrophic freshwater lake in eastern China, has experienced lake-wide toxic cyanobacterial blooms annually during summer season in the past decades. Spatial changes in the abundance of hepatotoxin microcystin-producing and non-microcystin producing *Microcystis* populations were investigated in the lake in August of 2009 and 2010. To monitor the densities of the total *Microcystis* population and the potential microcystin-producing subpopulation, we used a quantitative real-time PCR assay targeting the phycocyanin intergenic spacer (PC-IGS) and the microcystin synthetase gene (*mcyD*), respectively. On the basis of quantification by real-time PCR analysis, the abundance of potential toxic *Microcystis* genotypes and the ratio of the *mcyD* subpopulation to the total *Microcystis* varied significantly, from 4.08×10^4 to 5.22×10^7 copies/mL, from 5.7% to 65.8%, respectively. Correlation analysis showed a strong positive relationship between chlorophyll-*a*, toxic *Microcystis* and total *Microcystis*; the abundance of toxic *Microcystis* correlated positively with total phosphorus and *ortho*-phosphate concentrations, but negatively with TN:TP ratio and nitrate concentrations. Meanwhile the proportion of potential toxic genotypes within *Microcystis* population showed positive correlation with total phosphorus and *ortho*-phosphate concentrations. Our data suggest that increased phosphorus loading may be a significant factor promoting the occurrence of toxic *Microcystis* bloom in Lake Taihu.

Key words: microcystin-producing *Microcystis*; real-time PCR; toxic cyanobacterial bloom; microcystin synthetase gene cluster
DOI: 10.1016/S1001-0742(11)60745-6

Introduction

The occurrence of toxic cyanobacteria blooms in eutrophic freshwater ecosystem worldwide has increased during recent decades and it have became a global public health and environmental concern due to production of a large variety of secondary metabolities cyclic heptapeptide toxins called microcystins (MCs) (Carmichael et al., 2001; de Figueiredo et al., 2004). MCs are potent inhibitors of eukaryotic protein phosphatases 1 and 2A (MacKintosh et al., 1990; Dawson, 1998), they can cause severe liver damage and even death to animals and humans (Carmichael, 2001; Azevedo et al., 2002). There are more than 60 microcystin variants have been identified (Sivonen and Jones, 1999). Microcystin producing strains have been reported from several filamentous and unicellular genera including *Anabaena, Planktothrix*, and *Microcystis* (Dow and Swoboda, 2000).

It is a common phenomenon that toxic and non-toxic strains usually co-exist in many water bodies (Joung et al., 2010; Davis et al., 2009; Yoshida et al., 2007). However, it is almost impossible to distinguish toxic species from non-toxic species using traditional light microscopy because different strains of the same species show identical morphologies. It is well known that microcystin are synthesized nonribosomally by an integrated peptide-polyketide synthetase system encoded by the microcystin synthetase gene cluster (*mcy*) (Nishizawa et al., 1999, 2000; Tillett et al., 2000). Therefore, it is believed that the presence or absence of the *mcy* gene cluster can be used to distinguish MC-producing and non-MC-producing strains of cyanobacteria (Nishizawa et al., 2000; Hisbergues et al., 2003). Accordingly, many primers targeting the *mcy* gene cluster have been designed to distinguish MC-producing cyanobacteria. But the quantitative assessment of strain composition in water samples has long time been impeded, mainly because of a lack of appropriate methods.

Quantitative real-time PCR is one of the best methods for quantifying potentially toxic and non-toxic genotypes (Kurmayer and Kutzenberger, 2003; Vaitomaa et al., 2003; Rinta-Kanto et al., 2005; Furukawa et al., 2006). Previous documents have reported that there are spatial and temporal changes in the abundance of toxic and non-toxic

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genotypes and the proportion of toxic genotypes in different freshwater ecosystem. For example, Kurmayer and Kutzenberger (2003) estimated the proportion of toxic genotypes within colonies of *Microcystis* to be 1%–38% in samples collected from Lake Wannsee (Germany), while in Lake Mikata (Japan) potentially toxic genotypes formed 0.5%–35% of the total *Microcystis* population and it is positively related to nitrate concentration (Yoshida et al., 2007). The proportions of toxic genotypes varied considerably over time and between different sites (ranging from 6% to 93%) in Grangent storage reservoir (France) (Briand et al., 2009). Thus far, no studies have been published concerning the spatial variation in the abundance of potential microcystin-producing *Microcystis* in Lake Taihu.

Therefore, the aim of this study was to investigate the spatial variation in the abundance of toxic and non-toxic *Microcystis* genotypes in Lake Taihu during summer bloom period. We performed two independent real-time PCR assays to evaluate the community composition of *Microcystis* genotypes. One assay was used to quantify the total *Microcystis* population using the phycocyanin intergenic spacer (*PC-IGS*) that was previously used to examine the total *Microcystis* numbers (Kurmayer and Kutzenberger, 2003). A second real-time PCR assay was used to quantitatively detect the potentially microcystin-producing genotypes using *mcyD*-specific primers (Rinta-Kanto et al., 2005). In addition, the toxic subpopulation proportion in related to environmental factors were monitored through field investigation of *Microcystis* blooms in Lake Taihu.

1 Materials and methods

1.1 Sites description and water sampling

Lake Taihu is located at 30°05′N–32°08′N and 119°08′E–122°55′E, which is a large shallow hypertrophic lake (surface area: 2338 km^2, mean depth: 1.9 m) in eastern China that is the primary drinking water source for 30 million residents in the lake basin and Shanghai (the largest city in China) (Ye et al., 2009). It is also used heavily for boating, swimming and fishing. With the rapid development of economy and intensive use of water resources as well as industrial pollution in the lake basin, Lake Taihu has been in a eutrophic state since the 1980s, and it is currently considered to be hypertrophic. In addition, lake-wide cyanobacterial blooms dominated by *Microcystis* spp. has occurred annually in summer season in the last few decades (Chen et al., 2003). In this study, sampling was performed at eight sites distributed widely across the Lake Taihu (Fig. 1). Sampling sites N2 and N5 are located in Meiliang Bay and Zhushan Bay, respectively, which are two of the most hypereutrophic parts of Lake Taihu. Site N4 is located in Gonghu Bay that is a mesotrophic bay in the northeast part of the lake, with 10%–95% of its area covered with abundant submerged plants. Sampling sites W2 and W4 are located in the west lake area and sites S2 and S4 are located south and central areas of the lake, respectively. In August of 2009 and 2010, integrated

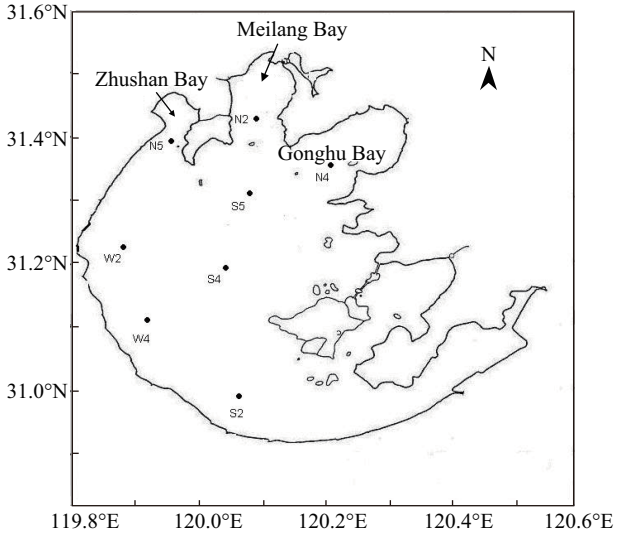

Fig. 1 Eight sampling locations in Lake Taihu are indicated by black circle and named with W2, W4, S2, S4, S5, N2, N4 and N5, respectively.

water samples were taken using a 2-m long and 10-cm diameter plastic tube from each site. Cells used to extract DNA for real-time PCR analysis were collected by filtering onto 47 mm diameter, 0.2 μm nominal pore-size polycarbonate membrane filters (Millipore, USA), which were immediately frozen at –20°C until processing.

1.2 Environment factors

Water temperature (Tem), dissolved oxygen (DO), pH and turbidity (Tur) were measured *in situ* by a multi-parameter water quality sonde (YSI6600, Yellow Spring Instrument, USA). For dissolved inorganic nutrients, nitrate (NO_3^--N) and orthophosphate (PO_4^{3-}-P), 50 mL water sample was filtered through a glass fiber filter (GF/F, Whatman, UK) and analyzed using a continuous flow system (San^{plus} analyzer, Skalar, the Netherlands). Total nitrogen (TN) and total phosphorus (TP) were analyzed according to the standard methods (Huang, 2000).

Water column chlorophyll-*a* concentrations were used as a proxy for phytoplankton biomass. To determine the chlorophyll-*a* concentrations, water samples were filtered using filter paper (GF/C Whatman, UK), then chlorophyll-*a* was extracted using 90% acetone and measured using fluorescence spectrophotometer (RF-5301, Shimadzu, Japan) (Cao et al., 2005).

For dissolved MCs analysis, 200 mL of water samples was filtered in duplicate using GF/C filters (Whatman, UK), the filtrate was concentrated according to standard methods (GB/T20466-2006). MCs were determined by high performance liquid chromatography with photodiode array detection (Agilent1200, Agilent, USA) equipped

Table 1 Primer sets used in real-time PCR in this study

Amplification sequence	Primer	Primer sequence
mcyD	F2	5′-GGTTCGCCTGGTCAAAGTAA-3′
	R2	5′-CCTCGCTAAAGAAGGGTTGA-3′
PC-IGS	188F	5′-GCTACTTCGACCGCGCC-3′
	254R	5′-TCCTACGGTTTAATTGAGACTAGCC-3′

with an ODS column (Ageilent Eclipse XDB-C18, 5 μm, 4.6 mm ×150 mm), using a gradient of 30% to 70% (*V*/*V*) acetonitrile (with 0.05% (*V*/*V*) trifluoroacetic acid) at a flow rate of 1 mL/min. MCs were identified using their typical UV spectra. Total microcystin concentrations were quantified as the sum of all microcystin microcystin peaks using MC-LR, -RR, and -YR standards (Sigma, Germany).

1.3 DNA extraction

DNA isolation was performed according to the protocol of Rinta-Kanto (Rinta-Kanto et al., 2005), followed by purification on QIAamp DNA MiniKit (Qiagen, USA). The purified DNA was used as template for real time PCR analysis.

1.4 Quantitative real-time PCR

Real-time PCR assays were used to quantify two genetic elements, the *PC-IGS* and *mcyD* regions. External standards used to determine the *PC-IGS* and *mcyD* copy numbers were prepared using the genomic DNA *Microcystis aeruginosa* strain PCC7806 offered by the FACHB Collection (Freshwater Algal Culture Collection of Institute of Hydrobiology, China). Cells from a known volume of the *M. aeruginosa* 7806 culture was filtered through 0.22-μm-pore-size filters, and the DNA extraction was as described above. The DNA concentration ($ABS_{260\ nm}$) and purity ($ABS_{260\ nm}/ABS_{280\ nm}$) were determined spectrophotometrically. Copy numbers of both genes on the basis of DNA were evaluated by Vaitomaa et al. (2003). A 10-fold dilution series of the DNAs was prepared and amplified with the *PC-IGS* and *mcyD* gene real-time PCR assays.

The real-time PCR assay was performed in a volume of 25 μL containing 12.5 μL 2× SYBR Green realtime PCR Master Mix (TaKaRa, Japan), 2.0 pmol each primer, 1 μL DNA from a standard strain or 2 μL DNA from lake water sample, and was adjusted to a final volume of 25 μL with sterile ultra-pure water. The assay was performed on the *Mastercycler realplex*^{4} (Eppendorf German) with *Mastercycler realplex* software. Amplification was performed as follows: The first step was an initial preheating for 2 min at 95°C. For *PC-IGS*, the initial preheating step was followed by 40 cycles: 95°C for 20 sec, 58°C for 30 sec, and 72°C for 20 sec. For *mcyD*, the initial preheating step was followed by 40 cycles: 95°C for 20 sec, 56°C for 30 sec, and 72°C for 30 sec. The melting temperature for the real-time PCR products was determined using the manufacturer's software. All tests were performed in triplicate.

1.5 Statistical analysis

The relationships between the *Microcystis* and the environmental factors were analyzed using a Pearson correlation coefficients. Before the correlation analysis, a logarithmic transformation was conducted for a few factors that were not normally distributed. All these analysis were done with the software SPSS 16.0 for windows.

2 Results

2.1 Environmental factors

Environmental factors (TN, TP, TN/TP ratio, PO_4^{3-}-P, NO_3^--N, DO, pH, turbidity, chlorophyll-*a*, and MC) of water samples collected in this study are shown in Fig. 2 and Table 2. During the study period, water temperature is 30.5°C and water quality is alkaline. The nutrient concentrations fluctuated spatially, in 2009, the TN and TP concentrations varied from 0.92 to 2.57 mg/L (average concentration: 1.66 mg/L) and from 0.034 to 0.2 mg/L (average concentration: 0.077 mg/L), respectively. In parallel, the TN and TP concentrations in 2010 ranged from 0.78 to 3.52 mg/L (average concentration: 1.68 mg/L) from 0.025 to 0.38 mg/L (average concentration: 0.11 mg/L), respectively. In addition, the concentration of chlorophyll-*a* in 2009 and 2010 ranged from 4.5 to 78 μg/L from 13.1 to 160.8 μg/L, respectively. It is evidence that cyanobacterial bloom is more intense in 2010 than in 2009. Meanwhile there are spatial changes in MC concentrations in 2009 and 2010, ranging from 0.36 to 0.85 μg/L, from 0.56 to 1.61 μg/L, respectively. Other physic-chemical parameters also varied significantly in different lake areas.

Table 2 Chlorophyll-*a* and MCs concentrations in Lake Taihu in August of 2009 and 2010

Month-Year	Sampling site	Chl-*a* (μg/L)	MC-RR (μg/L)	MC-YR (μg/L)	MC-LR (μg/L)	Total microcystin (μg/L)
August 2009	W2	51.7	0.11	0.01	0.32	0.44
	W4	19.7	0.32	0.01	0.02	0.36
	S2	10.0	0.06	<0.01	0.45	0.51
	S4	4.5	0.14	<0.01	0.50	0.64
	S5	17.2	0.19	<0.01	0.47	0.66
	N2	96.5	0.34	0.01	0.37	0.72
	N4	13.3	0.07	0.19	0.32	0.58
	N5	78.5	0.16	0.01	0.68	0.85
August 2010	W2	160.8	0.16	0.02	0.46	0.64
	W4	38.7	0.51	0.02	0.04	0.56
	S2	28.1	0.18	0.01	1.42	1.61
	S4	61.5	0.18	<0.01	0.66	0.84
	S5	13.1	0.25	<0.01	0.63	0.88
	N2	111.9	0.32	0.01	0.34	0.67
	N4	108.2	0.12	0.33	0.56	1.01
	N5	127.7	0.21	0.01	0.87	1.08

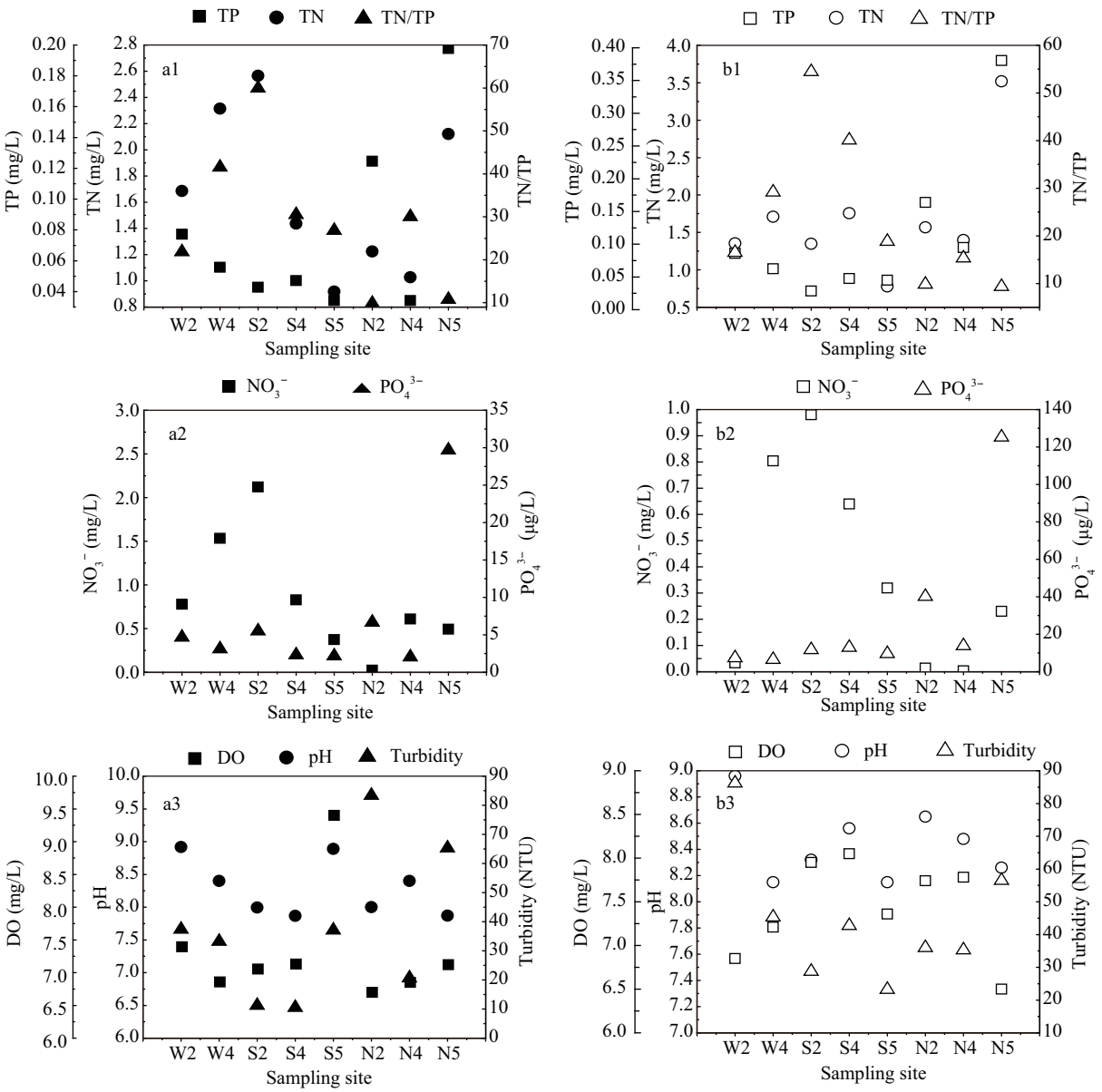

Fig. 2 Environmental variables of water samples in Lake Taihu in August of 2009 (a) and 2010 (b).

2.2 Standard curve for real-time PCR

The real-time PCR data show log linear relationships using both the *PC-IGS* and *mcyD* gene copies when the genomic DNA from *M. aeruginosa* PCC7806 was used as a template (Fig. 3). The detection range of *PC-IGS* copy numbers was from 2.2×10^2 to 2.2×10^7 copies in the reaction mixture; and the detection range of *mcyD* copy numbers was from 1.6×10^1 to 1.6×10^6. The melting curves of *PC-IGS* and *mcyD* real-time PCR products showed a peak at approximately 88.4 and 84.5°C, respectively, corresponding to the melting temperature of the *M. aeruginosa* standard strain (data not shown), which demonstrated the reliability of the real-time PCR amplification.

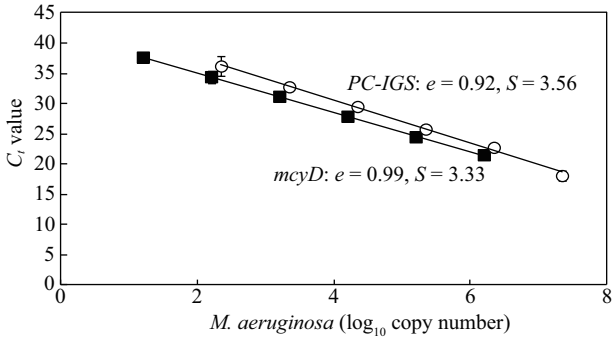

Fig. 3 Standard curves obtained by the *PC-IGS* gene and *mcyD* real-time PCR assays with the *M. aeruginosa* strain PCC7806 as a function of gene copy numbers. Each data point shows the threshold cycle (C_t) of standard DNA samples performed in triplicate. Amplification efficiency was calculated as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

2.3 Variations in abundance of toxic and non-toxic *Microcystis*

In 2009, the highest copy numbers of total *Microcystis* genotypes with the *PC-IGS* gene was detected at the Zhushan Bay (Fig. 4a). At other sampling sites, the abundance was 1–2 orders of magnitude lower. The abundance of subpopulation with the *mcyD* genotypes varied between 4.1×10^4 and 8.3×10^6 copies/mL. The highest and

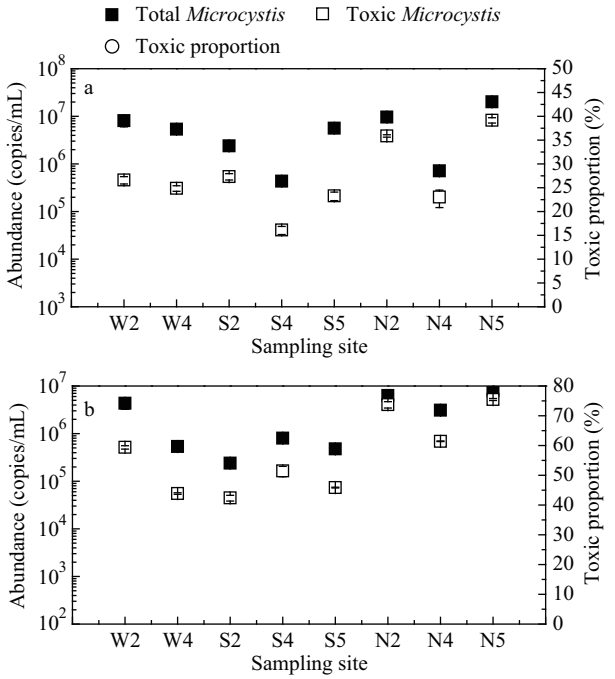

Fig. 4 Abundance of the total *Microcystis* and the *mcy* subpopulation in Lake Taihu in August of 2009 (a) and 2010 (b). DNA copies per milliliter were determined using real-time PCR. The toxic proportion is the relative DNA copy numbers of the *mcy* subpopulation compared to the total copy number of *Microcystis*. Error bars represent the standard deviations in triplicate.

lowest abundance of toxic genotypes were detected at Zhushan Bay and the center area, respectively. On the basis of the percentages calculated using the quantities of total *Microcystis PC-IGS* and toxic *Microcystis mcyD* in water samples, the toxic subpopulation proportion of total *Microcystis* was 3.8%–41.1%. In 2010, copy numbers of all *Microcystis* genotypes with the *PC-IGS* gene varied between 2.4×10^6 and 7.9×10^7 copies/mL (Fig. 4b), the toxic *Micorcystis* population with *mcyD* genotypes in every sample ranged from 4.5×10^5 to 5.2×10^7 copies/mL the ratio of the copy numbers of *mcyD* genotypes to the total *Microcystis* was 10.3%–65.8%.

2.4 Correlation analysis

The correlation of the abundance of toxic and total *Microcystis* genotype populations with environmental factors was evaluated (Table 3), and it revealed that there is a strong positive relationship between chlorophyll-*a* concentration, the toxic and the total *Micorcystis* population. Meanwhile it was also found that the toxic subpopulaton proportion correlated positively with TP and PO_4^{3-}-P concentrations ($P < 0.01$), but PO_4^{3-}-P is the only environmental factor that correlated positively with microcystin concentrations.

3 Discussion

Two important implications arise from the results of this study. First, a tiered response to a potentially toxic cyanobacterial bloom was demonstrated to be a practical approach to monitoring these events. The combination use of HPLC analysis, conventional PCR (results are not shown), and then quantitative PCR allowed us to rapidly and reliably detect and characterize this bloom event. Second, the results of this study confirm previous research that not all strains of *Microcystis* found in natural samples are capable of producing toxins (Tillett et al., 2001).

In the late 1980s, cyanobacterial bloom were common in the Meiliang Bay, one of the most trophic parts of Lake Taihu (Chen et al., 2003). But reductions in external phosphorus loading implemented in the mid-1990s reduced cyanobacterial biomass (Lin, 2002). By the late 1990s, water quality had improved substantially, and algal biomass had decreased in waters (Chen et al., 2003). Since 2000, cyanobacterial blooms composed primarily of *Microcystis* spp., have once again increased in frequency and scale, and this has been considered a sign of returning eutrophic conditions in the lake (Zhu, 2008). During the study period, the bloom of cyanobacteria was persisting in most sampling locations. This was confirmed through measuring high chlorophyll-*a* concentrations and high abundance of *Microcystis* populations in water samples.

In both years, various concentrations of microcystin, determined using HPLC analysis, were detected at all sampling sites. But it is important to note that the toxin data presented here represent estimates from the dissolved fraction in water samples. Given the dogma that most toxins are maintained within cells (Park et al., 1998), the toxin content within the particular phase (i.e., intracellular)

Table 3 Correlation coefficients among environmental factors, *Microcystis* population and microcystin concentration

	Chl-*a*	Total M	Toxic M	Ratio	MC	TN	TP	TN/TP	PO_4^{3-}	NO_3^-	pH	Tur	DO
Chl-*a*	1.00												
Total M	0.82**	1.00											
Toxic M	0.86**	0.87**	1.00										
Ratio	0.54*	0.74**	0.81**	1.00									
MC	0.18	0.20	0.28	0.28	1.00								
TN	0.24	0.48	0.41	0.44	–0.01	1.00							
TP	0.63**	0.82**	0.78**	0.80**	0.21	0.69**	1.00						
TN/TP	–0.66**	–0.60*	–0.62**	–0.48	0.04	0.07	–0.60*	1.00					
PO_4^{3-}	0.65**	0.80**	0.89**	0.80**	0.51*	0.55*	0.82**	–0.44	1.00				
NO_3^-	–0.66**	–0.53*	–0.55*	–0.39	–0.27	0.36	–0.38	0.86**	–0.38	1.00			
pH	0.31	0.24	0.10	–0.23	–0.14	–0.24	–0.15	–0.12	–0.10	–0.30	1.00		
Tur	0.77**	0.42	0.59**	0.28	0.05	0.09	0.45	–0.59*	0.36	–0.56*	0.17	1.00	
DO	–0.24	–0.21	–0.21	–0.31	0.20	–0.45	–0.39	0.17	–0.19	–0.11	0.45	–0.25	1.00

Chl-*a*: Chlorophyll-*a*; total M: total *Microcystis* population; toxic M: toxic *Microcystis* population; Tur: turbidity; ratio: the toxic subpopulation proportion; MC: microcystin concentration. *$P < 0.05$; **$P < 0.01$.

was not determined in this study. As such, estimates of microcystin toxicity in this article represent the minimum estimate.

Our results demonstrate that in Lake Taihu, microcystin-producing *Microcystis* genotypes generally coexist with non-toxic genotypes. In the entire *Microcystis* population, the proportion of microcystin-producing *Microcystis* cells did not exceed 70% of the total *Microcystis* abundance in any of the samples analyzed, the value is lower than 50% in most sampling locations. Our results generally agree with observations from previous studies, in spite of the use of different sampling approaches and obtaining samples from different locations, in that the relative abundance of potentially toxic *Microcystis* commonly remains well below 100% of total *Microcystis* abundance in natural populations (Kurmayer and Kutzenberger, 2003; Yoshida et al., 2007; Kardinaal et al., 2007a; Rinta-Kanto et al., 2009), indicating that a higher proportion of nontoxic *Microcystis* could be a more general phenomenon. In contrast, the dominance of a toxic subpopulation was observed in Lake Volkerak, the Netherlands (Kardinaal et al., 2007b) and Lake Ronkonkoma, US (Davis et al., 2009). In addition, the results of several competition experiments in laboratory between toxic and non-toxic strains have not produced a consistent conclusion (Briand et al., 2008; Schatz et al., 2005; Kardinaal et al., 2007b). Thereby, it is apparently impossible to explain these contradictory results using a single mechanism, the different results are likely to depend on a combination of the strain characteristics and environmental factors.

In the present study, the influence of environmental factors on *Microcystis* was investigated using a correlation analysis (Table 3). The abundance of toxic *Microcystis* genotypes showed significant positive correlation between TP and PO_4^{3-}-P, and negative correlation with TN/TP ratio and NO_3^--N. Furthermore, the percentage of potentially toxic genotypes were also positively correlated with TP and PO_4^{3-}-P, suggesting that increasing phosphorus concentrations can increase both the abundance as well as the the proportion of toxic *Microcystis* genotypes. Our observations agree best with those from a study conducted in a large eutrophic lake in America (Rinta-Kanto et al., 2009), where a positive correlation with the total abundance of *Microcystis* and microcystin-producing *Microcystis* was found. Lab and field experiments also showed that an increase in phosphorus concentrations elevated the growth rate of toxic *Microcystis* much more than nontoxic *Microcystis* (Vézie et al., 2002; Davis et al., 2009). In contrast with our findings regarding the *mcy*-gene copy abundance, Yoshida et al. (2007) found a positive correlation between the abundance of *mcyA*-copies and nitrate but no correlation with *mcyA* abundance and *ortho*-phosphate concentrations in a Japanese lake. However, Joung et al., (2010) found the proportion changes of potentially toxic *Microcystis* genotypes were only related with the water temperature in a Korea Reservoir. Therefore, these main factors controlling the internal dynamics of *Microcystis* population vary strongly between different environments.

One important caveat is that the role of biotic factors has not been account for in this study. For example zooplankton and amoebae grazing may affect the total biomass of cyanobacteria and *Microcystis* as well as the relative abundance of *Microcystis* genotypes because grazers may also exhibit selectively grazing depending on whether the cell is producing toxicn or not (Gobler et al., 2007; Wichelen et al., 2010). It is thus possible that some parmaters associated with grazing may have correlated with toxic versus non-toxin producing genotypes. As part of future efforts, this aspect of *Microcystis* ecology should no doubt be further investigated.

In conclusion, the measurement of toxic *Microcystis* using a molecular technique facilitated the differential monitoring of the dynamics of non-toxic and potentially toxic *Microcystis*. This is the first large-scale and multi-year study offering insight into factors influencing the *Microcystis* population in Lake Taihu. The results of our study suggest that in Lake Taihu increased phosphorus loading from autochthonous or allochthonous sources may be the main factor for the recurring *Microcystis* blooms in recent years.

Acknowledgments

This work was supported by the National Basic Research Program (973) of China (No. 2008CB418000). We are grateful to Dr. Min Zhang, Dr. Yuhong Jia and Dr. Yuanfeng Cai for their help regarding samples collecting.

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