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RESEARCH ARTICLE

Application of PCR and real-time PCR for monitoring cyanobacteria, *Microcystis* spp. and *Cylindrospermopsis raciborskii* in Macau freshwater reservoir

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Abstract Freshwater algal blooms have become a growing concern world-wide. They are caused by a high level of cyanobacteria, predominantly *Microcystis* spp. and *Cylindrospermopsis raciborskii*, which can produce microcystin and cylindrospermopsin, respectively. Long-time exposure to these cyanotoxins may affect public health, thus reliable detection, quantification, and enumeration of these harmful algae species has become a priority in water quality management. Traditional manual enumeration of algal bloom cells primarily involves microscopic identification which limited by inaccuracy and time-consumption. With the development of molecular techniques and an increasing number of microbial sequences available in the Genbank database, the use of molecular methods can be used for more rapid, reliable, and accurate detection and quantification. In this study, multiplex polymerase chain reaction (PCR) and real-time quantitative PCR (qPCR) techniques were developed and applied for monitoring cyanobacteria *Microcystis* spp. and *C. raciborskii* in the Macau Storage Reservoir (MSR). The results showed that the techniques were successful for identifying and quantifying the species in pure cultures and mixed cultures, and proved to be a potential application for water sampling in MSR. When the target species were above 1 million cells/L, similar cell numbers estimated by microscopic enumeration and qPCR were obtained. Further quantification in water samples indicated that the ratio of the estimated number of cell by microscopy and qPCR was 0.4–12.9 for cyanobacteria and 0.2–3.9 for *C. raciborskii.* However, *Microcystis* spp. was not observed by manual enumeration, while it was detected at low levels by qPCR, suggesting that qPCR is more sensitive and accurate. Thus the molecular approaches provide an additional reliable monitoring option to traditional microscopic enumeration for the ecosystems monitoring program.

Keywords cyanobacteria, *Microcystis* spp., *C. raciborskii*, microscopy, PCR and real-time PCR

1 Introduction

Eutrophication in bodies of freshwater, caused by a discharge of excessive nutrients, particularly nitrogen and phosphorus, is a growing concern. Under favorable conditions, it can lead to a proliferation of harmful cyanobacteria, such as *Microcystis* spp. and *Cylindrospermopsis raciborskii*. Cyanobacterial blooms can deteriorate the water quality by reducing transparency, decreasing biodiversity, releasing taste and odor-causing compounds, and of most concern, can produce cyanotoxins that pose a serious health hazard for humans (Paerl and Huisman, 2009). These cyanotoxins are known to be potent inhibitors of eukaryotic protein phosphatases 1 and 2A, causing changes in cytoskeletal proteins (Kurmayer and Kutzenberger, 2003). The World Health Organization (WHO, 1998) set the drinking water quality guideline of 1.0 μg/L as the microcystin-LR equivalent (Falconer et al., 1999). Furthermore, oxygen depletion and ammonium release caused by the decay of cyanobacteria can cause ecosystem disruption, such as killing fish.

Microcystis spp. and *Cylindrospermopsis* spp. (mainly *C. raciborskii*) are the representative species of microcystin- and cylindrospermopsin-producing cyanobacteria that commonly appear in aquatic ecosystems around the world (Ohtani et al., 1992; Falconer et al., 1999). Detection

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and quantification of potentially toxic species found in environmental samples are key for monitoring harmful algal blooms in addition to the subsequent development of remediation strategies. Moreover, the percentage of *Microcystis* spp. or *C. raciborskii* to cyanobacteria is an important indicator for forecasting potential corresponding cyanotoxins. Hence monitoring the cyanobacteria is of extreme importantance in freshwater reservoirs.

Cyanobacteria species have traditionally been identified on the basis of their microscopic morphology, physiology, and staining characteristics, which are inadequate and inaccurate, leading to misidentification due to their similarities. Moreover, quantification of species with similar morphology in environmental samples has to date, not been possible. Even skilled and experienced technicians are often unable to identify and enumerate the species in such complex samples. For example, *Microcystis* strains usually form scum (Oh et al., 2012) and thus *Microcystis* spp. to *Aphanocapsa* spp. is often misidentified (Wilson et al., 2000). In the same way, *C. raciborskii* has similar morphology to other cyanobacteria, such as *Anabaenopsis* and *Raphidiopsis*, making their quantification more difficult (Hawkins et al., 1997). In addition, manual cell enumeration is tedious and time consuming, leading to a delay in taking measures for cyanobacterial control.

Over the past few years, advances have been made in the molecular methods used for evaluating microbial diversity in natural environments enabling us to develop new techniques for identification and quantification of microorganisms. Early studies (Scholin et al., 1994) used the fluorescence in situ hybridization (FISH) approach coupled with microscopy or flow cytometry to count the number of cells of strain-specific harmful algal species. Recently, polymerase chain reaction (PCR) based on housekeeping genes, 16S/23S ribosomal RNA sequence, and *rpoC1* database have become available for researchers to use rDNA-targeted hybridization for studying cyanobacteria. The advantages of phylogenetic methods compared with the traditional identification and quantification methods are as follows. i) Molecular methods are more accurate: unlike morphology, physiology, and staining characteristics, conserved phylogenetic identity does not change over time or under different conditions. ii) These methods can yield target-specific quantitative data. By targeting multiple cyanobacteria, multiplex PCR, consisting of multiple primer sets in a single PCR mixture, can be developed (Al-Tebrineh et al., 2012). To quantify the cyanobacteria, real-time PCR, a further advancement of the basic PCR technique, is used. The SYBR green intercalating assay (Wittwer et al., 1997) and the TaqMan hybridization probe system (Giglio et al., 2003) are the two most frequently quantitative PCR (qPCR) techniques used, in which quantification of gene copy numbers is determined during the exponential phase of the amplification, when the amount of amplified target is proportional to the starting template. The quantification arises by measuring the fluorescence intensity, thus the amount of amplified DNA product at each stage during the qPCR cycle can be determined. The PCR and qPCR have been increasingly applied to monitor potential cyanobacteria population changes in diverse aquatic ecosystems worldwide (Kurmayer and Kutzenberger, 2003; Pearson and Neilan, 2008). These techniques have been subsequently applied for the detection and quantification of toxic genotypes, *mcy* genes and *cyr* gene of *Microcystis* spp. and *C. raciborskii*, respectively (Rasmussen et al., 2008; Ha et al., 2009), as they have been found to correlate with microcystin and cylindrospermopsin concentrations in pure cultures and environmental samples.

The Macau Storage Reservoir (MSR), the main storage reservoir for drinking water in Macau, has been experiencing algal bloom in recent years, with a high level of cyanobacteria, particularly *Microcystis* spp. and *C. raciborskii,* by microscopy. Considering the drawbacks of the traditional methods that are currently used, and the increasing use of molecular methods for the water quality monitoring program worldwide, PCR and qPCR were developed and applied in this study to identify and quantify *Microcystis* and *C. raciborskii*, and then potentially be applied to the water samples in MSR. Previous studies (Nübel et al., 1997; Otsuka et al., 1998; Rasmussen et al., 2008) have used the qPCR method in freshwater reservoirs to quantify toxic cyanobacteria that cause algal blooms, yet only one cyanobacteria genus or species was quantified. The purpose of this study was to develop and apply PCR (including mutiplex and real-time PCR) to simultaneously monitor the spatial and temporal distribution of cyanobacteria, *Microcystis* spp. and *C. raciborskii*, which are the dominant blooming causative species in MSR. To our knowledge, this is the only innovative study that integrates the multiplex PCR and real-time PCR for identifying and quantifying *Microcystis* spp. and *C. raciborskii* simultaneously in a freshwater reservoir. The results were then compared to those estimated by microscopic enumeration, and will subsequently be used for the development of the MSR water quality monitoring program.

2 Materials and methods

2.1 Cyanobacterial strains and culturing

The axenic strains of *Microcystis aeruginosa* (FACHB-905) and *C. raciborskii* (FACHB-1096) were obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Wuhan, China. Strains were cultivated and grown as batch cultures in BG11 media (Sigma-Aldrich) at 25°C and under irradiance of 1000 lx. Both strains were harvested during the stationary phase for performing PCR and qPCR experiments in pure cultures and mixed cultures.

2.2 MSR and field samples collection

2.2.1 MSR

The MSR (113°33′12″E longitude and 22°12′12″N latitude), located in the east part of the Macau peninsula, is the largest reservoir in Macau, with a capacity of approximately 1.9 million m^3. It receives raw water from the Pearl River of China, and can provide the entire water supply for all areas of Macau for approximately one week. This is of particular importance during the periods of high tide when the temporary water source contains a high salinity concentration caused by intrusion of sea water at the water intake location. Our recent study (Zhang et al., 2013) showed that MSR has a Tropic State Index (TSI) of 58–72 in 2010 and 64–82 in 2011, which is categorized as a eutrophic or hyper-eutrophic reservoir, and with a high level of cyanobacteria of 40–200 million cells/L detected in summer. The problems the reservoir has experienced instigated by the cyanobacterial blooms continue to intensify. Over the last five years, surveys taken by the Macao Water Co. Ltd. of the cyanobacteria, based on microscopy, found detectable levels of microcystin and cylindrospermopsin in the dominant species of *Microcystis* and *Cylindrospermopsis,* most of which are *C. raciborski*. The surveys were mainly based on the microorganisms' morphologies, which include identification and quantification of different species of cyanobacteria.

2.2.2 Field samples collection

Water samples were collected monthly in MSR from September 2011 to January 2012. Six sampling points were selected at three stations (Fig. 1) at two different water depths (0.5 m and 3.5 m below the water surface) each. Stations S1 and S3 are located in the inlet and outlet, respectively, while Station S2 is at the center of MSR. P1 (P2), P3 (P4), and P5 (P6) are defined as the sampling points at 0.5 m (3.5 m) below the water surface at Station S1, S2, and S3, respectively. Two liters of each water sample were collected and stored at –80°C for later analysis.

2.3 Enumeration of cyanobacteria using microscopy

Pure cultures (10–100 μL) or well-mixed water samples (1 mL) were preserved by adding one drop of Lugol's iodine before sedimentation for 72 h. The sludge samples were stored at room temperature. The strains of cyanobacteria present in sedimentation chambers, including *Microcystis* and *Cylindrospermopsis*, were counted manually by an inverted microscope using a Sedgwick-Rafter chamber from the method of McAlice (1971). At least three transects per chamber were screened to enumerate cyanobacteria strain at ×100 magnification, according to morphological criteria. The total cyanobacteria were estimated as the sum of all the different species of cyanobacteria.

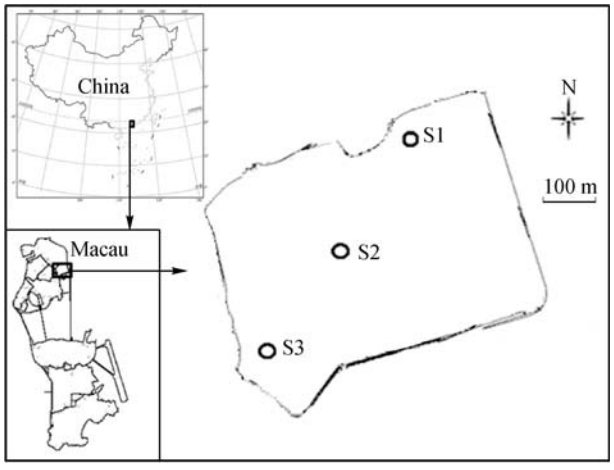

Fig. 1 Layout of MSR and location of water sampling points.

2.4 DNA extraction

Densely grown pure cultures (1 mL) or 175 mL of well-mixed water samples were taken and centrifuged in 3,000 rpm for 10 min to obtain the cyanobacteria pellets. Considering that the buoyant cells, such as Microcystis, do not settle easily at even a high speed of centrifugation, filtration followed by scrapping the cells from the filter paper was used. DNA extraction from the remaining pellets was carried out using QIAGen DNeasy Plant Mini Kit (Cat.No.69104, QIAGen, MD), according to the manufacturer's instructions. Extracted DNA was stored at –80°C.

2.5 PCR

Genomic DNA templates of pure cultures, mixed cultures, and water samples were amplified by the GeneAmp® PCR system 9700 (Applied Biosystems, CA) to demonstrate the presence of cyanobacteria, *Microcystis* spp., and *C. raciborskii* using specific primer sets (Table 1). Primer specificities, with reference to the selected sequences in this study, were verified using the BLAST program of the NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Each PCR mixture contained 0.5 μL of a DNA template solution (0.5–5 ng DNA/50 μL PCR), 5 μL of 10×PCR Buffer ($MgCl_2$ plus), 4 μL of the dNTP mixture, 0.5 μL (10 pmol) of each primer and 0.25 μL of *TaKaRa Taq*™ [20 mM Tris–HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween20, 0.5% Nonidet P40, 50% Glycerol solution] (TaKaRa Biotechnology, China), and was adjusted to a final volume of 50 μL with sterile water (Sigma, USA). The PCR was performed as follows:

95 °C for 5 min, 30 cycles at 95 °C for 30 s, 50 °C –60 °C for 30 s and 72 °C for 1 min, and a final extension step at 72 °C for 7 min. Gel electrophoresis was run to confirm the presence of cyanobacteria, *Microcystis* spp. and *C. raciborskii* in the samples. All PCR products were separated on 2% agarose gels and observed on Molecular Imager ChemiDoc XRS System (Bio-Rad, USA) after staining with ethidium bromide for 20 min.

2.6 Multiplex PCR

Multiplex PCR was performed for simultaneous detection of *Microcystis* and *C. raciborskii*, in mixed cultures and water samples, using the corresponding primers (Table 1). Each multiplex PCR mixture contained 1 μL of DNA template solution (0.5–5 ng DNA/50 μL PCR), 25 μL of Multiplex PCR Mix 2, 0.5 μL (10 pmol) of each primer and 0.25 μL of Multiplex PCR Mix 1, according the manufacture (TaKaRa Biotechnology, China), and was adjusted to a final volume of 50 μL with sterile water (Sigma, USA). The multiplex PCR was performed as follows: 94 °C for 1 min, 30–40 cycles at 94 °C for 30 s, 50 °C for 90 s and 72 °C for 90 s, and a final extension step at 72 °C for 10 min. All PCR products were separated on 4% agarose gels and observed on Molecular Imager ChemiDoc XRS System (Bio-Rad, USA) after staining with 6× loading dye for 20 min.

2.7 Real-time PCR

Real-time PCR were performed in the ABI 7500 Real-Time PCR system (Applied Biosystems, CA). All reactions were carried out in a total volume of 50 μL, containing 26 μL SYBR®*Premix Ex Taq*™ (Tli RNaseH Plus), including *TaKaRa Ex Taq* HS, dNTP Mixture, Mg^{2+}, Tli RNaseH, SYBR® Green I, plus ROX Reference Dye II (DRR420, TaKaRa Biotechnology, China), 1 μL forward primers and 1 μL reverse primers (Table 1), 18 μL deionized water and 4 μL DNA templates. The thermal protocol for *Microcystis* spp. was that first warming 2 min at 50°C, and then preheating 10 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 34 s at 50 °C, then 1 min at 72 °C, and finally dissociated 15 s at 95 °C, 1 min at 64 °C and 15 s at 99 °C. Fluorescence was measured at the end of each cycle at 72 °C through channel F1 (530 nm) and a heating rate of 20 $°C \cdot s^{-1}$. For cyanobacteria and *C. raciborskii* qPCR, thermal cycling steps were nearly the same as that for *Microcystis*, except for the annealing temperature that changed from 50 °C to 60 °C. All samples were amplified in triplicate. After qPCR amplification, fluorescent melting curve analysis was performed by gradually increasing the temperature from 72 °C to 95 °C at a rate of 0.1 $°C \cdot s^{-1}$. A correlation between the gene copy numbers and the threshold cycle number (Ct) (the cycle number at which the fluorescence exceeds the threshold) can be obtained.

2.8 Real-time PCR standard curves and detection limits

Standard curves and the detection limits for real-time PCR were established using 10-fold dilutions of the extracted pure culture DNA, with the concentrations ranging from 10^{13} to 10^{4} cells/L. Corresponding primers (Table 1) were applied to the pure cultures of *Microcystis* spp., and *C. raciborskii*. Specific primers were used for targeting the pure culture of *C. raciborskii*, and the cell numbers were estimated as the *C. raciborskii* equivalent cells/L. The standard curves can be developed to relate Ct values to the cell numbers estimated by microscopy counting.

It noted that, as the cyanobacteria cell number directly reflects the level of algal blooms situation, we tried to relate the cell numbers to the Ct values, instead of relating the gene copy number to the Ct values, assuming the gene copy number in each cell is constant. The cell numbers estimated by real-time qPCR in the present study can be compared to that counted by microscopy which is currently used in the Macau Water Utility. Thus the standard curves developed here were based on the cell numbers in the pure cultures, not on the gene copy number, even though the gene copy number was directly measured in the qPCR. From this point of view, the cyanobacteria cell amounts, rather than their gene copy number, was estimated directly from the Ct values in the qPCR without quantification of cyanobacteria gene copy number.

Table 1 Primers used for PCR and real-time PCR assays

Target	Primer	Sequence(5′–3′)	DNA length	Ref.
Cyanobacteria 16S rDNA	CYA359F	GGGGAATYTTCCGCAATGGG	470 bp	(Nübel et al., 1997)
	CYA781R(a)	GACTACTGGGGTATCTAATCCCATT		
	CYA781R(b)	GACTACAGGGGTATCTAATCCCTTT		
Microcystis 16S rDNA	MSR-S1f	TCAGGTTGCTTAACGACCTA	409 bp	(Otsuka et al., 1998)
	MSR-S2r	CTTTCACCAGGGTTCGCGAC		
C. raciborskii rpoC1	cyl2	GGCATTCCTAGTTATATTGCCATACTA	308 bp	(Rasmussen et al., 2008)
	cyl4	GCCCGTTTTTGTCCCTTTCGTGC		

3 Results and discussion

3.1 Pure cultures

3.1.1 PCR

The PCR amplification results (P1–P3, Fig. 2) confirmed the feasibility of using cyanobacteria 16S rDNA, *C. raciborskii rpoC1* genes and *Microcystis* 16S rDNA for detection of the pure cultures of cyanobacteria, *C. raciborskii*, and *Microcystis* spp., with corresponding bands of 470 base pairs (bp), 310 bp and 400 bp shown by gel electrophoresis, respectively. These results were consistent with the primers' DNA design lengths (Table 1). Before performing each qPCR, the gel electrophoresis results of real-time PCR products were used to confirm the presence of cyanobacteria, *C. raciborskii*, and *Microcystis* spp. (T1–T6, Fig. 2).

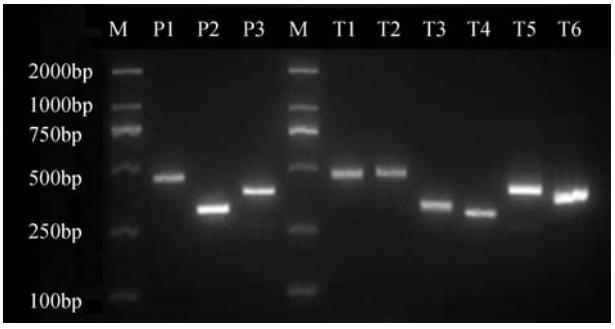

Fig. 2 PCR amplification results of pure cultures tests for cyanobacteria 16S rDNA (P1), *C. raciborskii rpoC1* genes (P2), and *Microcystis* 16S rDNA (P3); verification of real-time PCR products of standard curve development for cyanobacteria 16S rDNA (T1 and T2), *C. raciborskii rpoC1* genes (T3 and T4) and *Microcystis* 16S rDNA (T5 and T6); M represented marker.

3.1.2 qPCR

After applying the appropriate amount of amplified DNA with the corresponding primers for targeting the specific class, species, and strains, the copy number of the corresponding genes can be qualified with the Ct value. The standard curves were developed to relate the Ct values to the cell numbers estimated by microscopy. Table 2 shows the standard curves developed for cyanobacteria, *C. raciborskii* and *Microcystis* strains versus the Ct numbers, which have high correlation coefficients ($>99\%$). For example, the regression equation was $y = 49.255–2.994x$ ($R^2 = 0.993$, $p < 0.0001$) for rDNA of *C. raciborskii*, where y is the Ct and x is amount of initial DNA concentration as represented as the $\log_{10}$ (cell numbers). The cyanobacteria cell number estimated using the standard curves was expressed as *C. raciborskii* equivalent cells/L. The estimates of the slopes, 3.00 and 3.51, were similar to the theoretical value of 3.32 ($1/\log_{10}2$), which is an exact double value for each polymerization cycle (Larionov et al., 2005). To further test the reliability of these standard curves, two independent tests for each pure culture strain were conducted by comparing the cell numbers estimated using qPCR with those counted manually. Similar cell numbers with the approximate population ratio of 0.52 to 1.95 were obtained (Table 3), suggesting that the standard curves established in our study were able to estimate the cell number of cyanobacteria, *C. raciborskii* and *Microcystis* spp. in pure cultures. Furthermore, using *C. raciborskii* targeted by cyanobacteria primers is successful in quantifying the cyanobacteria abundance. However, speical care must be taken when applying this quantification method to cyanobacteria, as its different growth phases may affect the DNA copies of the cell number (ranging from 3 to 100) (Vaitomaa et al., 2003).

The sensitivity analysis in this study confirmed the high sensitivity of the qPCR using SYBR Green, which can be used to detect the lowest units of several cells per milliliter of total cyanobacteria. By diluting the initial DNA amount to the appropriate folds, the sensitivity of qPCR is approximately 50 cells/mL of *C. raciborskii*, and around 140 cells/mL of *Microcystis* spp. Our reliable detection limits were similar to those obtained by Moreira et al. (2011), in which the detection for cyanobacteria achieved up to 11 cells/mL and for *C. raciborskii*, reached a limit of 258 cells/mL.

3.2 Mixed cultures

3.2.1 PCR and qPCR

Mixed cultures of *M. aeruginosa* and *C. raciborskii* were used to verify specificity of the primer sets targeted for *C. raciborskii rpoC1* and *Microcystis* 16S rDNA. Pure cultures were mixed in three ratios, 9∶1, 1∶1, and 1∶9 for both species. PCR results (Fig. 3) confirmed the presence of *C. raciborskii rpoC1* (M1–M3) and *Microcystis* 16S rDNA (M4–M6) in a mixture of both species, indicating the specificity of the primers used for mixed cultures. A comparison of the cell numbers, estimated using microscopy and qPCR (Table 3), showed that the difference was very small, with an approximate ratio of 0.94∶1.16, further proving that the selected primer sets were highly specific in mixed culture conditions.

3.2.2 Multiplex PCR

By using specific primers targeted for *Microcystis* spp. and *C. raciborskii* together, the multiplex PCR can be used for simultaneous detection of both cyanobacteria species in mixed cultures, as well as in the water samples (Fig. 4). Compared with single PCR, multiplex PCR can be used to

Table 2 Standard curve parameters from real-time PCR for the cyanobacteria, *C. raciborskii* and *Microcystis* spp.

Target	Slope	*y*-intercept	r^2
Cyanobacteria 16S rDNA	–1.656	33.951	99.2%
C. raciborskii rpoC1	–2.994	49.255	99.3%
Microcystis 16S rDNA	–3.510	44.262	99.6%

Table 3 Comparison of cell numbers of specific gene targets as estimated by counting and real-time PCR in pure cultures and mixed cultures

	Primer target	Pure cultures strains used	Counting number/(cells·L^{-1})	Real-time PCR results/(cells·L^{-1})
Pure cultures	Cyanobacteria 16S rDNA	*C. raciborskii*	3.7×10^{11}	2.9×10^{11}
			1.8×10^{11}	1.5×10^{11}
	C. raciborskii rpoC1	*C. raciborskii*	2.8×10^{7}	5.4×10^{7}
			1.2×10^{8}	1.9×10^{8}
	Microcystis 16S rDNA	*M. aeruginosa*	3.7×10^{5}	1.9×10^{5}
			1.8×10^{5}	1.8×10^{5}
Mixed cultures	*C. raciborskii rpoC1*	*C. raciborskii* : *M.aeruginosa* (9 : 1)	1.1×10^{8}	1.1×10^{8}
		C. raciborskii : *M.aeruginosa* (1 : 1)	6.0×10^{7}	6.4×10^{7}
		C. raciborskii : *M.aeruginosa* (1 : 9)	1.2×10^{7}	1.2×10^{7}
	Microcystis 16S rDNA	*M.aeruginosa* : *C. raciborskii* (9 : 1)	3.3×10^{5}	3.0×10^{5}
		M.aeruginosa : *C. raciborskii* (1 : 1)	1.9×10^{5}	1.8×10^{5}
		M.aeruginosa : *C. raciborskii* (1 : 9)	3.7×10^{4}	3.2×10^{4}

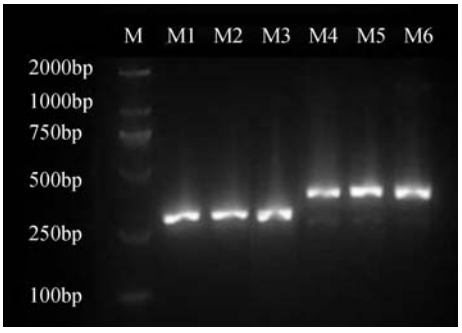

Fig. 3 PCR amplification results of primers' specificities targeted for *C. raciborskii rpoC1* genes with *C.raciborskii/M. aeruginosa* = 9 : 1 (M1), *C.raciborskii/M.aeruginosa* = 1 : 1 (M2), and *C.raciborskii/M.aeruginosa* = 1 : 9 (M3); for *Microcystis* 16S rDNA with *M.aeruginosa/C.raciborskii* = 9 : 1 (M4), *M.aeruginosa/C.raciborskii* = 1 : 1 (M5), and *M.aeruginosa/C. raciborskii* = 1 : 9 (M6); M represented marker. *C.raciborskii* primers were used for lanes M1–M3, and *Microcystis* primers were used for lane M4–M6.

detect multiple genes rapidly and simultaneously from a number of cyanobacteria species in a single reaction.

3.3 Water samples

3.3.1 PCR

The PCR products shown in Fig. 5 confirm the presence of cyanobacteria, *C. raciborskii* and *Microcystis* spp. at the sampling point of 0.5 m from the water surface in Station S1 (P1). Similar results were also observed for those in other sampling points (not shown here). Moreover, the multiplex technique was also shown to be feasible when applied to water samples (Fig. 4). Multiplex PCR provides such advantages as reduction in assay time, labor, and chemical cost. However, careful primer design (especially considering the DNA oligomer melting temperature T_m), with extensive optimization of reagents and condition, was required to avoid non-specific primer-dimer interactions and to obtain a well-balanced set of amplicons (Schoske et al., 2003; Al-Tebrineh et al., 2012).

3.3.2 qPCR

By using qPCR, the cell numbers of cyanobacteria, *Microcystis* spp. and *C. raciborskii* were estimated and showed that higher levels of cyanobacteria species were detected by qPCR than by manual counting, with concentrations of approximately 1,290,000–210,150,000 cells/L (counting) versus 1,410,000–395,000,000 cells/L (qPCR) for cyanobacteria, and 820,000–84,000,000 cells/L (counting) versus 665,000–152,000,000 cells/L (qPCR) for *C. raciborskii* (Fig. 6). It was noted that the cell numbers of cyanobacteria and *C. raciborskii* estimated by qPCR results were close to those estimated by manual counting, with the ratios of 0.4–12.9 and 0.2–3.9, respectively. All these data suggest that qPCR can be applied to water samples for quantifying the abundances of

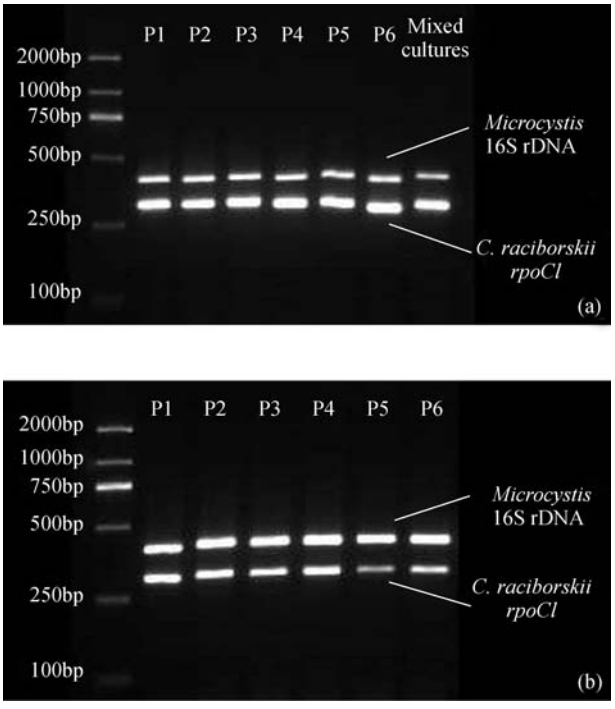

Fig. 4 Multiplex PCR results of mixed cultures and water samples in November (a) and water samples in January (b) targeted for *C. raciborskii rpoC1* genes and *Microcystis* 16S rDNA.

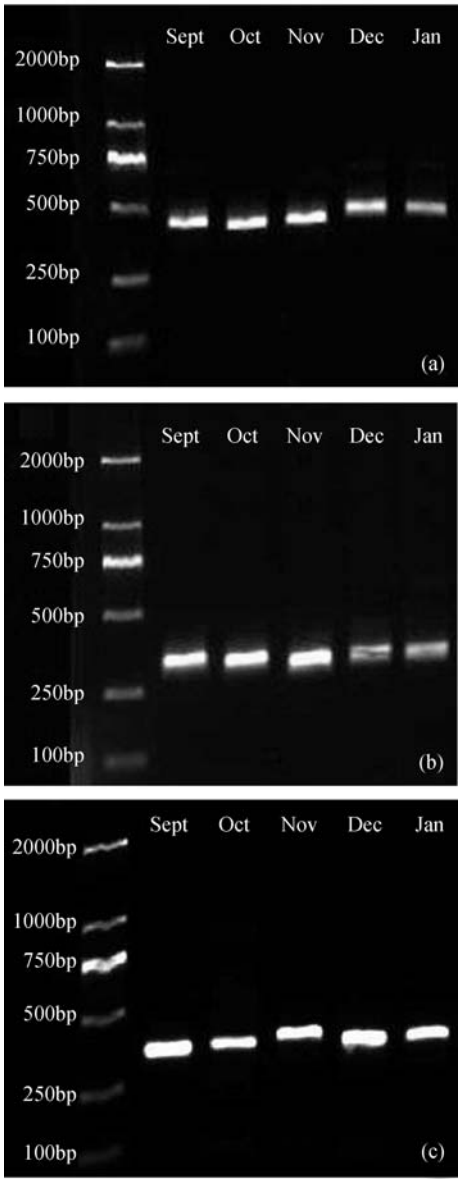

Fig. 5 Amplification results of P1 water samples for cyanobacteria 16S rDNA (a), *C. raciborskii rpoC1* genes (b) and *Microcystis* 16S rDNA (c) from September to January.

cyanobacteria and *C. raciborskii*. However, it was interesting to note that no *Microcystis* spp. was observed in all 30 water samples using microscopy due to the low number of *Microcystis* cells, while a low level of 214,000–2,480,000 *Microcystis* cells/L were detected using qPCR. These qPCR results were consistent with the PCR results shown above, implying that the qPCR method is more sensitive and accurate than manual counting, where one cell was counted in the microscopic image after dilution was converted to 195,000 cells/L in the original water samples.

These results reveal that the qPCR method developed here can be used to quantify the *Microcystis* and *Cylindrospermopsis* at low concentrations, even when they cannot be detected by traditional counting. Similar cell counts were obtained using both methods when the *C. raciborskii* abundance in MSR exceeded approximately 1 million cells/L. However, this study observed that substantial differencees occur when the organism concentration is low; i.e., lower than 1 million cells/L of *Microcystis*. The cell enumeration with microscopy seems to underestimate the abundance of *Microcystis*, which is consistent with the findings by Tomioka et al. (2008), but contradicted the results by Artz et al. (2006) who found that dead cells in water samples can produce positive results by microscopy, leading to potential overestimation of the cell numbers. Thus the differences by both quantification methods should be carefully examined in the future. Furthermore, the relationship between cyanobacteria species abundance is not clearly understood by microscopy, due to the inaccurte measurement of cell abundance. Even though the qPCR method used in the present study cannot distinguish the dead cells from the viable cells, due to the persistence of DNA in the environment after cells lose viability, it is believed that this difficulty can be overcome by combining qPCR, with a photo-induced cross-linking technique that could inhibit PCR amplification of DNA from dead cells. This follows the assumption that the study of cell viability is important to increase our understanding of the problems caused by algal blooms. Thus, compared to traditional microscopic counting, qPCR should be more appropriate in monitoring cyanobacteria abundance in MSR.

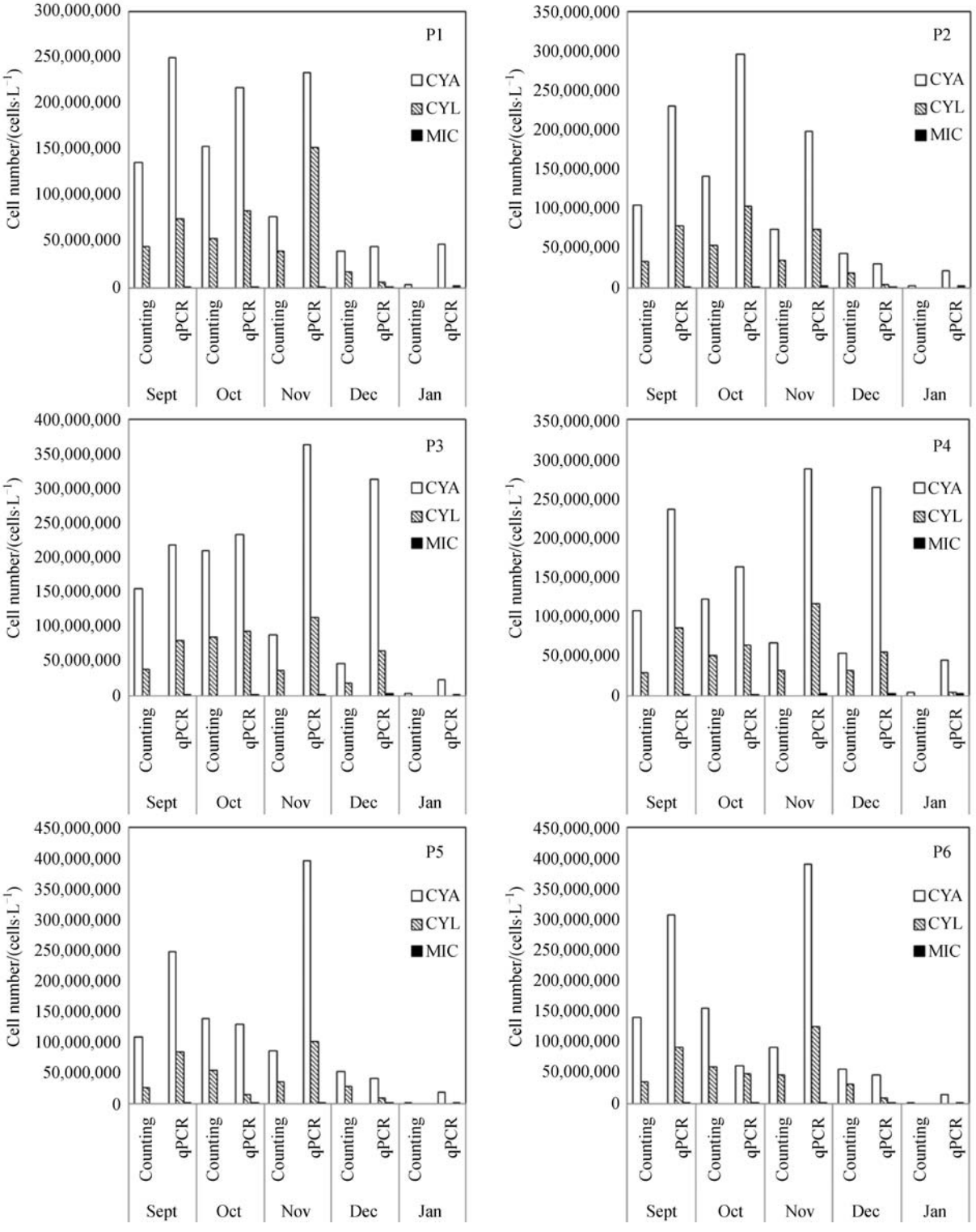

Fig. 6 Comparison of qPCR results and cell counts of water samples for cyanobacteria (CYA), *Cylindrospermopsis* (CYL) and *Microcystis* (MIC) in each sampling point (P1–P6).

By taking into consideration that strains of *C. raciborskii* were dominant in the MSR, the cyanobacteria quantified here were expressed as *C. raciborskii* equivalent cells/L based on the standard curve developed from the pure culture *C. raciborskii*, which needs to be carefully interpreted. Cyanobacteria frequently contain more than a single species in reservoirs. The gene copy numbers of cyanobacteria, based on 16S rDNA and amplified as an

expression of diverse species, leads to variations of cyanobacteria cell numbers estimated by qPCR.

Further statistical analysis, using the cluster analysis (D_{link}/D_{max}) < 20 (not shown here) indicates that there is no significant difference between cell numbers for each of the two sampling points. Since *Cylindrospermopsis* is a thermophilic species, it has a rapid growth rate and can become dominant during summer and autumn, yet can greatly decrease during the seasons that experience lower temperatures (Alster et al., 2010), which agrees with the qPCR percentage results shown in Fig. 7. The ratios of *C. raciborskii* over the total cyanobacteria estimated by both quantification methods (Fig. 7) were shown to be similar from September to November, whereas the ratios were dissimilar in December and January. This variance is due to the underestimation of cell numbers by microscopy MSR as described above. The ability to sensitively and accurately quantify the toxic strain presence in the water samples is the first step toward understanding the cyanobacteria dynamics and risks of algal blooms, although quantifying the abundance of potential toxin producers does not imply toxicity unless the toxins, such as microcystin and cylindrospermopsin, are actually measured.

Due to the short hydraulic retention time of MSR, it is believed that the communities and compositions of cyanobacteria are affected by the hydrodynamic parameters (such as water depth and rainfall) and physicochemical parameters (such as temperature, pH, nitrogen and phosphorus concentration, light intensity, and iron concentration) (Rinta-Kanto et al., 2005; Ha et al., 2009). To meet water quality regulations, cyanobacteria species, in addition to the cyanotoxins, microcystins and cylindrospermopsins must be routinely monitored. Hence, further studies will focus on quantifying different compositions of the cyanobacteria species, and measuring the cyanotoxins and environmental parameters in the reservoir, thus relating the environmental parameters to the abundance of different cyanobacteria specie populations and cyanotoxin levels.

The number of water bodies affected by cyanobacterial blooms has been increasing worldwide. In MSR, *Microcystis* spp. was previously the dominant bloom-forming cyanobacteria, yet as of 2011, *C. raciborskii* has become dominant. Even though the chief cause for this shift is not completely understood, it can be assumed that due to the fact that water is pumped into the MSR, the water quality, including the physical, chemical water parameters, and cyanobacteria community, and their interaction, is hence directly affected by the influents. For example, *C. raciborskii* is generally thought of as a tropical species due to its affinity for warm water temperatures (25°C–30°C) (Pidasak, 1997), common in Macau. Unfortunately, to our knowledge, there is no further available reference that provides a deeper explanation of the relationship of the species compositions and water quality parameters. It is

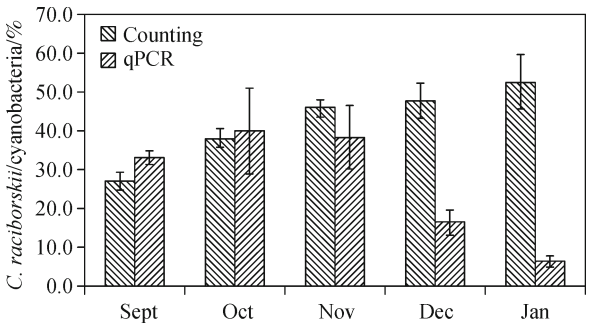

Fig. 7 Comparison of the percentages of *C. raciborskii* over cyanobacteria from September 2011 to January 2012 by cell count and qPCR. The percentages were taken as the average over the samples and the error bars represent the standard deviation of the six sampling points.

thus of critical importance for water monitoring utilities to rapidly detect and quantify these potentially toxic cyanobacteria to provide early detection and management of blooms in the reservoir by using innovative techniques. However, use of traditional microscopy is limited; for example, *Microcystis* strains usually form scum, which is difficult for enumeration, and *C. raciborskii* has numerous morphotype similarities, such as *Anabaenopsis*, *Raphidiopsis*, and *Cylindrospermum* (Scholin et al., 1994). In addition, it has been reported that until now, keys (morphological attributes) have not been developed for *C. raciborskii*, as a distinct species.

The current study has demonstrated the validity of PCR use for the detection of cyanobacterial strains in eutrophic water, with more accurate and sensitive detection results shown above. Compared with the PCR technique, traditional microscopy has the advantage of being cost effective, simple, with the ability to identify the presence of cyanobacteria. However, it is time-consuming, and is unable to distinguish between morphologically similar species. Alternatively, PCR is specific, sensitive, and reproducible with the ability for adaption to numerous data measurements simultaneously. The disadvantage of PCR is that it requires the knowledge of designing primers utilizing a gene sequence database, to target specific species. A sterile environment should be maintained during the PCR operation to avoid any contamination leading to amplification of non-specific products, which may affect PCR results (Felske and Osborn, 2005). Furthermore, water samples often contain complicated mixtures of organic matter which may inhibit PCR. In spite of these disadvantages, the molecular methods are still undoubtedly useful for early effective detection of cyanobacteria (Humbert et al., 2010). As PCR is only qualitative, the application of qPCR can provide a quantitative option for the assessment of cyanobacterial community, which correlates the gene copy number measured to the cells number. Orr et al. (2010) pointed out several possible reasons for the poor correlation

between qPCR results and cell enumeration, such as: 1) failure to sediment negatively buoyant cells during centrifugation prior to DNA extraction; 2) natural variability in gene cell quotas between different species and strains, including polyploidy; 3) genetic variation within the target regions of the primers at both strain and species levels; 4) sub-quantitative recovery of cells and DNA from samples; and 5) over- or underestimation of cell concentrations using traditional microscopy methods. Based on the manual count qPCR results, the integration of traditional microscopy with the PCR for routine monitoring programs for cyanobacteria will conceivably be developed in the future, with an early signal of dominant species identification and specific primer applications to quantify their concentrations in the reservoir to allow for subsequent monitoring.

The qPCR method developed in the present study must be standard and useable for all cyanobacteria, not only a few species. We picked up *Microcystis* and *C. raciborskii* here because they are the representative toxic species of microcystin- and cylindrospermopsin-producing cyanobacteria that commonly appear in aquatic ecosystems used as a drinking water resource. Detection and quantification of such potentially toxic species in environmental samples are key in studies for monitoring the harmful algal blooms as well as the subsequent development of remediation strategies. In addition, the percentage of *Microcystis* spp. or *C. raciborskii* to cyanobacteria is an important indicator in the forecast of potential of corresponding cyanotoxins.

Currently there is a decreased amount of consideration for cyanobacteria species in the Genebank data. Sequencing technologies, such as pyrosequencing or Ion Torrent PGM sequencing, can be used to determine the DNA sequences of the cyanobacteria species that are not included in the database. Based on the alignment of the sequences from the Genebank data, those cyanobacteria species primers can be designed, and thus used for targeting those species, using real-time PCR.

4 Conclusions

The MSR has been plagued with an algal bloom problem for many years, with *Microcystis* spp. and *C. raciborskii* as the dominant species. Traditional microscopic counting is currently being used in monitoring the cyanobacteria species, which is limited due to inaccuracy and time consumption. To overcome these limitations, PCR and qPCR methods were developed in this study. Our results showed that the techniques were proven to be successful for identification and quantification of cyanobacteria, *Microcystis* spp. and *C. raciborskii* in pure cultures, mixed cultures, and water samples, suggesting that promising technologies have been developed that can replace the microscopic counting method, particularly for early detection of algal bloom formation. Moreover, one benefit from our study was the development of a multiplex PCR that has the potential to discern if two or more dominant species are present in a specific water sample in a single PCR assay, which will assist the targeted users of the reservoir for further research and to promote public health in a rapid and accurate approach. The quantification in water samples indicated that similar cyanobacteria and *C. raciborskii* cell numbers estimated by both microscopy and qPCR were obtained, with ratios of 0.4–12.9 and 0.2–3.9, respectively. However, *Microcystis* spp. was not observed by manual counting, while it was detected at low levels by qPCR, thus ascertaining that the method is more sensitive and accurate than microscopic counting. Thus, these molecular approaches offer new tools to address many remaining questions in fundamental and applied cyanobacterial ecology. By considering the advantages shown here of PCR and qPCR over microscopy, these techniques, in addition to microscopic enumeration, can be potentially used for routine water quality monitoring in at-risk water bodies such as the MSR.

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